Chronic effects of single intra-peritoneal injection of endosulfan on rainbow trout (*Oncorhynchus mykiss*) and field observations of caged rainbow trout in Oshawa Creek.

Jeffrey Andrew Armour

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List of Abbreviations

ACh	acetylcholine
AChE	acetylcholine esterase
AhR	aryl hydrocarbon
ARNT	aryl hydrocarbon nuclear translocation protein
AtChI	acetylcholine iodide
BCF	Bioconcentration Factor
BSA	bovine serum albumin
CLOFMP	Central Lake Ontario Fisheries Management Plan
CLOCA	Central Lake Ontario Conservation Authority
CS	citrate synthase
CYP 1A1	cytochrome P-450 1A1
DFO	Department of Fisheries and Oceans
DHHS	United States Department of Health and Human Services
DMSO	dimethylsulfoxide
DO	dissolved oxygen
DTNB	5,5'-dithiobis (2-nitrobenzoate)
ELISA	enzyme linked immunosorbent assay
EPA	United States Environmental Protection Agency
EROD	7-ethoxyresorufin O-deethylase
GSI	gonadosomatic index
HPG	hypothalamic-pituitary-gonadal axis
HSI	hepatosomatic index
i.p.	intra-peritoneal
LC_{50}	concentration of chemical that causes 50 % mortality
LDH	lactate dehydrogenase

MFO	mixed function oxygenase
MS-222	tricane methanesulfonate
PBS	phosphate buffered saline
PMRA	Health Canada Pest Management Regulatory Agency
SPMD	semi-permeable membrane device
SSDH	Serum sorbitol dehydrogenase
TBPS	TWEEN-20 in phosphate buffered saline
TFM	3-Trifluoro-Methyl-4-Nitro-Phenol
TGA	thermogravimetric analysis
USGS	United States Geological Survey
VTG	vitellogenin
7-ER	7-ethoxyresorufin

1. Abstract

The organochlorine pesticide endosulfan has been shown to be highly toxic to fish and there is some evidence to support that it may act as an endocrine disrupting chemical. Juvenile rainbow trout (*Oncorhynchus mykiss*) were caged at 4 sites in Oshawa Creek during the fall and spring of 2008 and 2009 while another group was intra-peritoneal injected in the laboratory with varying concentrations (ppm) of endosulfan. Plasma vitellogenin (VTG) levels, liver ethoxyresorufin-O-deethylase (EROD), citrate synthase (CS), lactate dehydrogenase (LDH), and brain acetylcholine esterase (AChE) (caged fish only) enzymatic activities were measured. Trout injected with endosulfan experienced an increase of the anaerobic (LDH activity) and a decrease of the aerobic (CS activity) metabolic pathways, while male VTG levels increased. Since it was a singular injection, VTG results have to be confirmed. Fall caged trout showed increased EROD activity and inhibited AChE activity while those caged in the spring experienced an unexpected exposure to the lampricide 3-Trifluoro-Methyl-4-Nitro-Phenol (TFM) which disrupted metabolic parameters (inhibited CS and increased LDH activity). Both fall and spring caged trout experienced no induction of VTG activity. Further research is needed since the spring exposure was altered due to the unplanned TFM treatment and thus did not represent a valid temporal replicate.

2. Review of the Literature

2.1.0 Oshawa Creek

2.1.1 Oshawa Creek Watershed

The Oshawa Creek Watershed is located in Durham region of Southern Ontario, specifically in the city of Oshawa. The watershed originates in the Oak Ridges Moraine, North of Oshawa, extends through South Slope, Lake Iroquois Plain and ends at Oshawa Harbour on Lake Ontario (CLOFMP, 2007). It also extends into the Municipality of Clarington, Town of Whitby, and the Township of Scugog (CLOCA, 2002) (**Figure 2.1**). The watershed itself encompasses an area of approximately 120 km^2 with a length of 50 km and a water course of approximately 360 km (including all tributaries) (CLOFMP, 2007).

Oshawa Creek is divided into eight separate sub watersheds which include; West North, East North, West South, East South, Goodman Creek, Main Branch, Montgomery Creek, and harbour (CLOCA, 2002). The sub watersheds even though are all part of the Oshawa Creek Watershed, vary substantially in their physiographic composition (CLOCA, 2002).

2.1.2 Physiography and Surficial Geology of Oshawa Creek Water Shed

Physiography is the description of the land as controlled by the underlying rock and unconsolidated soil material. It is these qualities that help control the environmental conditions of the watershed, such as the hydrological cycle. The major components of the hydrological cycle are precipitation, evapotranspiration, infiltration to groundwater, surface runoff, and ground water discharged (**Figure 2.2**) (CLOCA, 2002). The

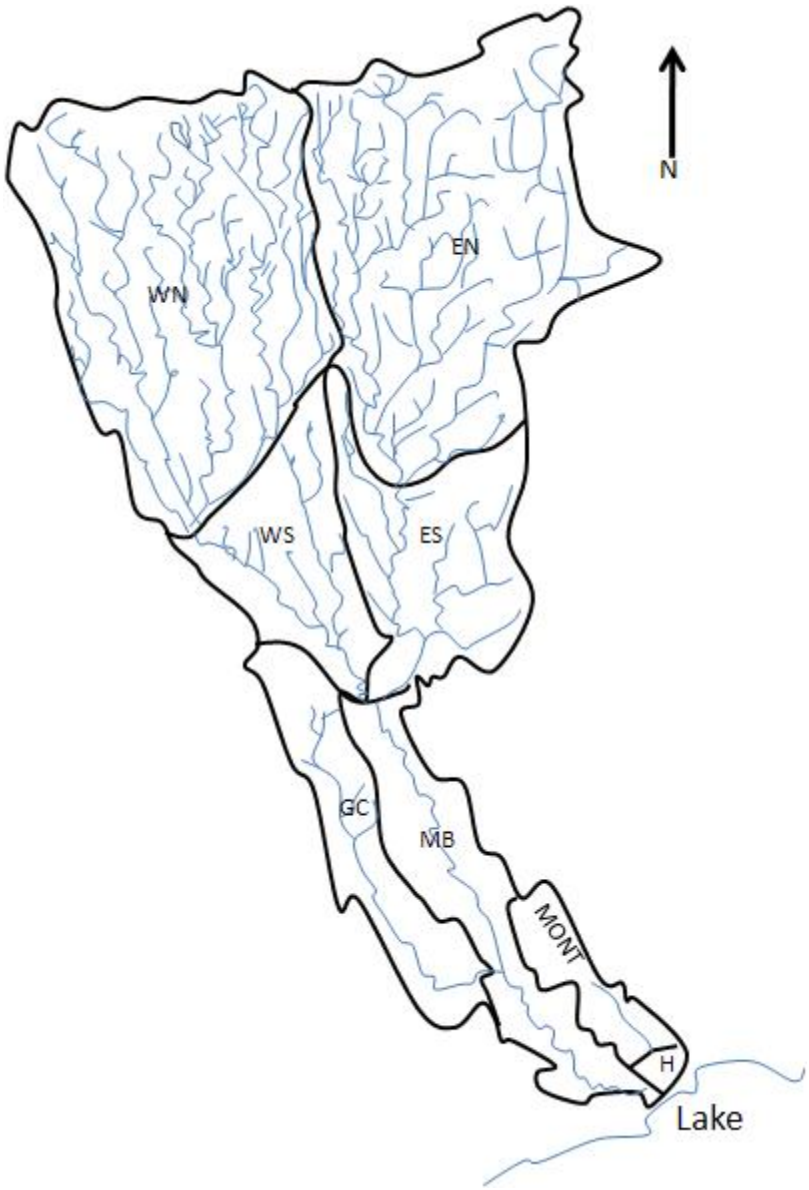

Figure 2.1: Oshawa Creek Watershed in Oshawa, Ontario from its spring fed mouth to its outfall into Lake Ontario. Oshawa Creek spans ~120 km^2 and has a total water course of ~360 km.

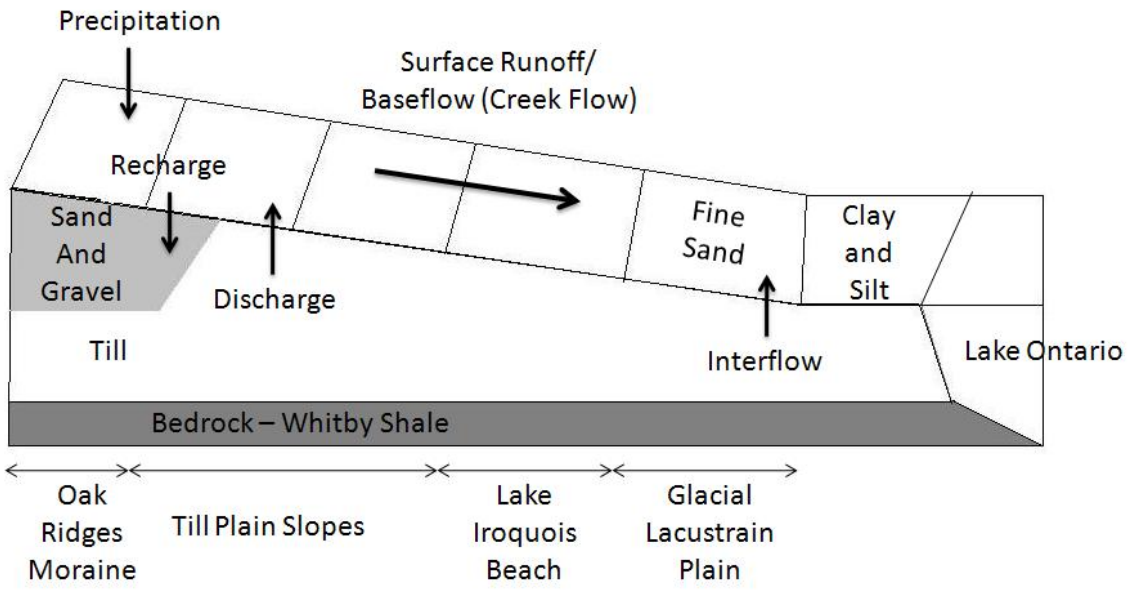

Figure 2.2: General surface hydrology of the Oshawa Creek Watershed.

hydrological cycle in conjunction with physiographic parameters affect the water flow within the watershed. The Oak Ridges Moraine is topographically hilly composed of sand and gravel deposits (CLOFMP, 2007). Hummocky topography creates ideal conditions for ground water infiltration and evapotranspiration. Almost all precipitation in this region is converted into groundwater, can penetrate to depths of up to 100 m and helps feed the tributaries in Oshawa Creek (CLOFMP, 2007).

South Slope is the largest physiographic portion of the watershed which extends from the Oak Ridges Moraine to Lake Iroquois. This section of the watershed is less diverse in altitude variation than compared to the Oak Ridges Moraine, with an average slope of 2 % (CLOCA, 2002). This regions surficial soil is primarily composed of sandy till materials (CLOFMP, 2007). This allows for a high percentage of precipitation to enter and remain in the soil, approximately 20 % (CLOFMP, 2007).

The last major section of the watershed is the Lake Iroquois Plain which is primarily a flat region with an average slope of about 1 % (CLOCA, 2002). This section is comprised of a 3 km wide band of sand and gravel beach bars along the northern edge of the plain which can extend to a depth of 8m (CLOCA, 2002). The abundance of sand and gravel allows for large amounts of water to infiltrate the area and then spread laterally to the valley regions as the downward movement of the water is inhibited by a layer of till (CLOCA, 2002). As the course of the water flows southerly in the watershed the different topographic regions migrate through several demographic regions including; agricultural, urban, and industrial.

2.1.3 Ecological Importance

Oshawa Creek is an ecologically important feature of the watershed as it provides habitat and food sources for numerous aquatic and terrestrial organisms. The river itself has a thermal gradient of warm-cold depending on the sub watershed. Focusing on the aquatic environment, the river supports breeding grounds for several migratory species including various Salmonids (salmon and rainbow trout, brown trout, and brook trout)

(CLOCA, 2002; CLOFMP, 2007). This is supported by a variety of cold water habitats that provide ideal conditions for breeding (CLOCA, 2002).

Besides Salmonids, Oshawa creek provides habitat for a variety of other species including; blacknose dace, creek chub, longnose dace, fathead minnow, rock bass, pumpkinseed, and slimy sculpin (CLOCA, 2002). In total there are approximately, 31 fish species representing 11 families (CLOFMP, 2007).

2.1.4 Land Use: Historical and Present

As previously mentioned Oshawa Creek flows through several different demographics before its outfall into Oshawa Harbour. These different demographics include; agricultural, urban, and industrialized zones (CLOCA, 2002; CLOFMP, 2007).

Historically, the Oshawa creek watershed was primarily sculpted by glacial movements (Wisconsin glacier) leaving predominant limestone ridges, with deep smooth concave-shaped valleys (CLOCA, 2002). It was this glacier that created the aforementioned regions within the Oshawa creek landscape. During the 1700s it was thought that Oshawa Creek was wider with greater water flow allowing flat bottomed boats to traverse the watershed (CLOCA, 2002). The area was also predominantly used for agriculture, which in the long run developed the community that is now Oshawa. The prime areas of agriculture were determined by the physiographic features, where most farming operations were clumped around areas with loamy soil that could drain well or Lake Ontario where there were decreased hazards due to frost (CLOCA, 2002). Agriculture practices extended to the edges of Lake Iroquois, where the soils were erosion prone and not favourable.

This type of activity continued until the 1800s when mass deforestation occurred to create farm land and produced a highly productive timber trade (CLOCA, 2002). Forest clearing continued until a decline in 1910. It was during this time that the first legitimate business (general store) opened, representing the beginning of Oshawa (CLOCA, 2002). With an increasing population and the availability of water, Oshawa

began to grow, with the location of the first general store becoming the present day commercial center of Oshawa (CLOCA, 2002).

Industrially, Oshawa has primarily been considered a “manufacturing town” with a large focus on the automotive industry. Before the automotive industry, other industries occupied Oshawa, and Oshawa Creek including; the Robson tannery (located on the West North sub watershed) and Warren Mill (located on the Main Branch sub watershed) (CLOCA, 2002).

As would be imagined, Oshawa is significantly more developed today than it was in the 1800s or even the early 1900s. Even with mass development in the area the predominant land usage still pertains to agriculture with 51 % allocated to crop land, and 18 % to pasture lands (CLOCA, 2002). Residential occupancy only accounts for 11 % of the total land use in Oshawa and along the watershed (CLOCA, 2002). Broken down further, Goodman Creek (901 hectares) land use is primarily residential (45 %), industrial/commercial (22 %), and crop/pasture land (19 %) (CLOCA, 2002).

The Main Branch sub watershed of Oshawa Creek is unlike the other areas in that it is almost completely developed (containing much of older Oshawa). Main Branch contains mostly residential and industrial/commercial development at 57 and 21 % respectively (CLOCA, 2002). The West South sub watershed of Oshawa Creek is comprised mainly of agricultural land (68 %), with residential and industrial and commercial only accounting for 2 and 3 % respectively (CLOCA, 2002). The West North sub watershed is primarily agricultural and pastured land (61 and 18 % respectively). Residential lands in this area only account for 4 % of the total sub watershed (CLOCA, 2002). East South sub watershed is also primarily crop land and pasture land accounting for 67 and 6 % of the total area of the sub watershed. Residential developments only account for 14 % of this area (CLOCA, 2002)

The Montgomery Creek sub watershed is in the heart of Oshawa and is completely developed with all its land use going towards residential, commercial, and industrial applications (CLCOA, 2002).

With such a large portion of the Oshawa Creek Watershed dedicated to agricultural practices, it is reasonable to assume that a wide range of different herbicides, pesticides, and fungicides are being used (Garret, 2004; Harris *et al.,* 2000). Among this mixture, one of the predominant pesticides being used in the area is endosulfan (Harris *et al.,* 2000).

2.2.0 Endosulfan

2.2.1 Chemical Composition and Introduction of Use

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) is a non-systemic organochlorine pesticide (insecticide/acaricide) commercially known as "Thiodan" (EPA, 2002; Bernabò *et al.,* 2008; Bayer Cropscience, 2008). Technical grade endosulfan is composed of two biologically active stereo-chemical isomers alpha and beta in a ratio of 70 % to 30 % respectively (Garret, 2004; EPA, 2002).

Despite the introduction of organochlorine pesticides in the 1950s (endosulfan in 1957) and many of them being banned in the 1970s (Siang *et al.,* 2007), endosulfan is still used commercially worldwide in developed and developing countries including Canada (Siang *et al.,* 2007, Garret, 2004, and Tuduri *et al.,* 2006). Endosulfan was thought to be a safer alternative than other organochlorine pesticides like DDT (dichloro-diphenyl-trichloroethane) because it had not been shown to cause eggshell thinning, and has a much shorter half life in the environment (Bernabò *et al.,* 2008; Benguira and Hontela, 2000).

2.2.2 Applications of Endosulfan

As previously mentioned endosulfan is a non-systemic (does not affect the body as a whole) insecticide, namely an acaricide (aphids, ticks and mites). Besides being used specifically for these organisms endosulfan can also be used for a wide range of other

organisms (Presibella *et al.,* 2005). A short list of organisms that endosulfan is capable of controlling include; meadow spittlebug, cucumber beetle, green stink bug, corn earworm, squash bug, thrips, grape phyloxera, grape leafhopper, leather leaf fern borer, aphids, rose chafer, lilac borer, and douglas fir needle midge (EPA, 2002; PMRA, 2007; Bayer Cropscience, 2008). The list of applicable crops and non crop vegetation include: barley, beans, brussels sprouts, cauliflower, collards, kale, corn, eggplants, oats, melons, rye, potatoes, squash, tomoatos, apples, almonds, nectarines, peaches, wheat, alfalfa, kohlrabi, Christmas trees, shade trees, and ornamental plants and shrubs (EPA, 2002; PMRA, 2007; Naqvi and Vaishnavi, 1993; Bayer Cropscience, 2008). With a large range of crop and non crop uses, it is quite realistic to assume a large potential for endosulfan to come into contact with non-target organisms and environments.

2.2.3 Mode of Action

Endosulfan's mode of action has not been conclusively proven, with two postulates being currently tested. The first postulate is that endosulfan affects the central nervous system by binding to the picrotoxinin site in the g-aminobutyric acid (GABA) chloride ionophore complex (Harris *et al.,* 2000; Markey *et al.,* 2007). GABA is an inhibitory neurotransmitter that operates through membrane polarization which is regulated through increased chloride flux (Harris *et al.,* 2000; Gant *et al.,* 1987; Markey *et al.,* 2007).

The second postulate is similar in fashion but deals with the inhibition of Ca^{+} and Mg-ATPase (Naqvi and Vishnavi, 1993; Yu, 2008). It is thought that this is only attributable to the stereo chemical specificity of α-endosulfan (Harris *et al.,* 2000). In both cases a negative after potential is created which prevents the axon from recovering (Harris *et al.,* 2000; Naqvi and Vaishnavi, 1993; Yu, 2008). This causes continual firing of neurotransmitters from a single stimulus leading to a depressed state followed by hyper activity, tremors, convulsions, rigid paralysis, respiratory failure and eventually death in non target organisms (Harris *et al.,* 2000; Naqvi; Vaishnavi, 1993 and Yu, 2008).

2.2.4 Use in Canada

Even though a majority of organochlorine pesticides have been banned since the 1970s in developed countries, there are a few (endosulfan, dicofol, and methoxychlor) still in use in the world today (Harris *et al.,* 2000). Endosulfan is one of these compounds even though it has been banned in other developed countries such as Germany, Sweden, United Kingdom, Netherlands, Colombia, and Singapore (Siang *et al.,* 2007). Canada has not yet followed suit in banning endosulfan but is in a period of reduction (14000 kg used in 1988, reduced to 3700 kg in 2003) (Tuduri *et al.,* 2006). See **Table 2.1** for sale reduction of endosulfan across Canada for the time period of 1991-2001. In a study done by Shen *et al.* (2005), passive air sampling devices were used to determine the concentration of organochlorine pesticides across North America. They found that the concentration of endosulfan in the air was generally lower than 158 pg/m^3. Across Canada, gaseous endosulfan ranged from 3.1-681 pg/m^3 for α-endosulfan and 0.03-119 pg/m^3 for β-endosulfan, with the highest concentrations found in Okanagan valley, British Columbia, East Point (PEI), McCreary (Manitoba), and Toronto (Ontario) (Tuduri *et al.,* 2006). This is of particular importance because atmospherically transferred pesticides can cause toxicity to non-target organisms hundreds of kilometres away.

2.2.5 Use in Durham Region, Ontario

As mentioned above, Toronto, Ontario had one of the highest atmospheric concentrations of endosulfan detected (Tuduri *et al.,* 2006). In Ontario alone, endosulfan use has decreased with a total consumption of 14000 kg in 1988, 6900 kg in 1998, and 3700 kg of active ingredient in 2003 (Tuduri *et al.,* 2006; Harris *et al.,* 2000). In 1993 the regions of heaviest use are in the Southern and Western portions of Ontario (Lambton, Kent, Elgin, Haldimand-Norfolk, Niagara, Durham, York and Simcoe counties) (Harris *et al.,* 2000).

Surface water concentrations in these regions have ranged from <0.01 to 0.54 mg/L which is of environmental significance since the Canadian safety guidelines for aquatic life has been set at 0.02 µg/L (Harris *et al.,* 2000; Berntssen *et al.,* 2008; Garret, 2004).

2.2.6 Methods of Application and Sources of Environmental Contamination

Generally speaking, there are three main methods used for endosulfan application; aerial spraying, boom spraying, and hand held application. Others sources include accidental and illegal release (Harris *et al.,* 2000; Hose *et al.,* 2003). Aerial spraying and boom spraying pose the greatest risk out of the three for environmental contamination as they produce the greatest amount of diversity for indirect transport and thus, will be focused upon (Garret, 2004; Hose, 2003).

Table 2.1: Amount of endosulfan sold (tons) across Canada between the years 1991 and 2001 (Tuduri *et al.*, 2006).

Year	1991	1995	1999	2000	2001
Region					
Prince Edward Island	Unavailable	Unavailable	10-50	<10	<10
New Brunswick	Unavailable	Unavailable	4.6	Unavailable	3.5
British Columbia	6.9	7.3	4.7	Unavailable	Unavailable

2.2.6.1 Aerial/Boom Spraying

Aerial/boom (a boom sprayer is a device attached to a tractor to spray fertilizer or pesticides on the desired crop) spraying can result in direct exposure to non-targeted areas by several means including; direct application on non-targeted areas and spray drift. Spray drift is unavoidable in these methods of application and weather conditions must be monitored to lower this risk (Garret, 2004). In aerial application, it has been observed that spray drift had caused an area 500 m downwind of application to receive a 14 % dose equivalent compared to the targeted field (Hose *et al.,* 2003).

While boom over spraying may not contaminate as large an area, it has the potential to contaminate an area more extensively, with the non-target area receiving a higher dose (Hose *et al.*, 2003; Lal, 2007). Both of these methods can then lead to other modes of environmental contamination.

2.2.6.2 Vapour Transport

Vapour transport is the process of volatilization of a chemical and transportation to other areas (possibly globally). A study by Kennedy *et al.* (2001) found that approximately 70 % of an endosulfan load sprayed onto a field of cotton had volatilized within the first two to three weeks. Unfortunately there is not much that can be done to avoid this except to not spray on exceedingly hot days (Hose *et al.,* 2000). Volatilized materials can enter a cycle of deposition and volatilization, known as the "grasshopper effect" (Shen *et al.,* 2005). It has been noted that organochlorine pesticides (DDT, aldrin, and endosulfan) are quite capable of accomplishing this (Gouin *et al.,* 2008; Yao *et al.,* 2008). Chemicals in this cycle essentially "hop" from area to area moving North or South (depending on the hemisphere) and eventually ending up in the poles (Gouin *et al.,* 2008). Endosulfan being a semi-volatile compound can enter this cycle and has been traced all the way to the arctic (410 pg/L in 1986) (EPA, 2002; Tuduri *et al.,* 2006). In this process the "hopping" movement can cause both aquatic and terrestrial contamination in areas along the way (Hose *et al.,* 2003; Yao *et al.,* 2008).

2.2.6.3 Run-off

Unlike spray drift, run-off contamination applies more to aquatic environments and generally causes a higher dose received but with less frequency (Hose *et al.,* 2003). This occurs when a pesticide is applied to a field before a storm event, which leads to pesticide bound soil particles entering the various aquatic ecosystems (Hose *et al.,* 2003; DeLorenzo *et al.,* 2002). Run-off events may also occur by the release of irrigation tailing water and irrigation itself (leaching of the chemical into ground water) (Hose *et al.,* 2003). These types of contamination can cause waterborne levels to exceed the median lethal concentrations for several aquatic species and can be raised above the maximum allowable water concentrations (Hose *et al.,* 2003; Capkin *et al.,* 2006; Glover *et al.,* 2007; EPA, 2002). The allowable concentration of endosulfan to protect wildlife in Canada is 0.02 µg/L (CCME, 2007), while water concentrations in Ontario have been <0.01-0.54 mg/L in the last two decades (Harris *et al.,* 2003). Higher reported concentrations are well above the 96 h LC_{50} (lethal concentration in which to kill 50 % of the sample group after 96 h) for several freshwater fish: bluegill sunfish (*Lepomis macrochirus*) (1.7 µg/L), fathead minnows (*Pimephales promelas*) (1.5 µg/L), and rainbow trout (*Oncorhynchus mykiss*) (1.75 µg/L) (EPA, 2002; Capkin *et al.,* 2006). Due to the low tolerance of many species, a small spill or agricultural runoff of endosulfan can cause adverse affects for aquatic systems.

2.2.7 Persistence in the Environment

Endosulfan has been shown to be moderately-highly persistent in the environment, depending on the media that it is in (EPA, 2002; Tuduri *et al.,* 2006; Siang *et al.,* 2007). In all media endosulfan will break down to its daughter compounds (endosulfan sulfate, diol, ether, lactone, and hydroxyether (**Figure 2.3**)) (Deger *et al.,* 2003). The breakdown of the parent compound is predominantly associated with oxidative and hydrolytic mechanisms with the predominant metabolite being endosulfan sulphate which can be equally toxic as endosulfan itself (Garret, 2004; Hose *et al.,* 2003).

The sections below will outline the persistence of endosulfan in three main media (sediment/soil, water, and air).

2.2.7.1 Sediment/Soil

There has been a lot of controversy over the persistence of endosulfan in the environment and particularly in sediments/soils. One factor that has been agreed upon is that the smaller the particle that endosulfan is bound to, the longer it will stay in the environment (Garret, 2004; Dorval *et al.,* 2003). Endosulfan bound to sediment will persist longer than in the other mediums, with up to 75 % of the compound in solution being bound to clay or silt like particles (Hose *et al.,* 2003). Endosulfan bound to sediment/soil is able to persist up to 2-6 years (Dorval *et al.,* 2003; EPA, 2002; Gormley and Teather, 2003; Raymond *et al.,* 2001). This is mainly due to the hydrolysis of α-endosulfan to endosulfan sulfate (the most persistent form of endosulfan) and the minor degradation of β-endosulfan (binds more tightly to the sediment than α-endosulfan) to endosulfan sulfate (Hose *et al.,* 2003; Garret, 2004). It is this metabolite that allows endosulfan to persist for longer periods of time in the environment when compared to the other mediums (Hose *et al.,* 2003; EPA, 2002).

When bound to neutral or acidic soils, α-endosulfan can last up to 2 months and β-endosulfan can last up to 2.5 years (EPA, 2002). Similar to sediment persistence, α-endosulfan breaks down first due to ease of volatility, with its major transformation product being endosulfan sulfate (Hose *et al.,* 2003; PMRA, 2007). β-endosulfan goes through the same process, just at a slower pace since it binds to the sediment more strongly (Garret, 2004; Hose *et al.,* 2003; PMRA, 2007).

In both sediment and soil, the initial oxidation/hydrolysis of endosulfan is quite quick, producing endosulfan sulfate (the major derivative product). It is endosulfan sulfate that predominantly remains and persists in the environment (EPA, 2002; PMRA, 2007; Deger, 2003).

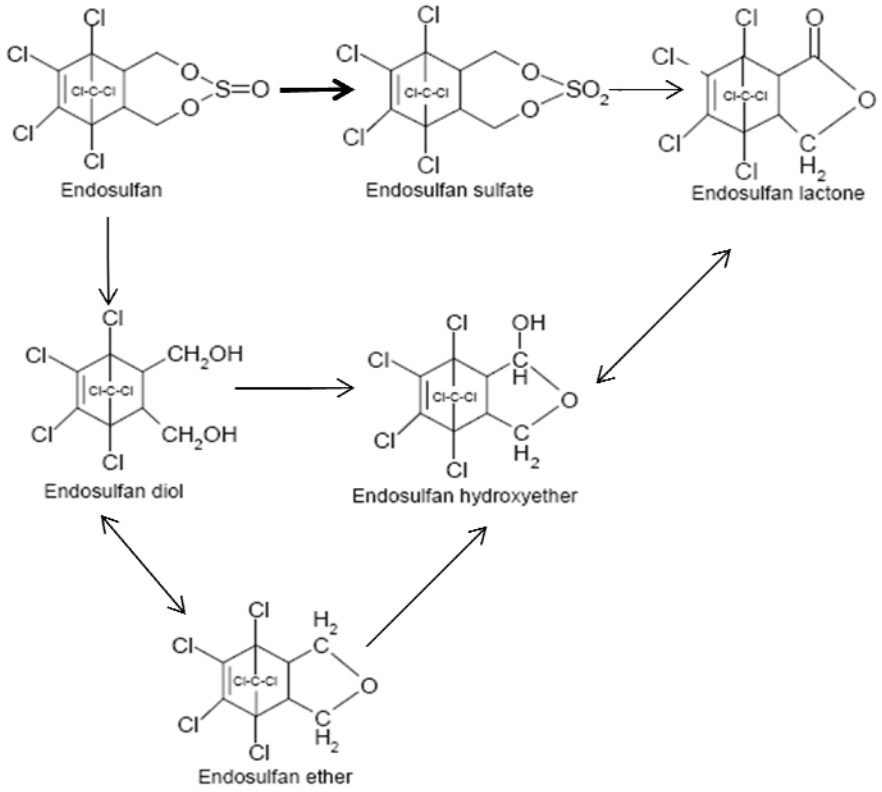

Figure 2.3: Chemical breakdown of endosulfan into its daughter compounds.

2.2.7.2 Water

Unlike its persistence in sediment/soil, endosulfan is not very persistent in the water column, especially under basic conditions (EPA, 2002; Garret, 2004). This could be attributed to its moderate water solubility of 0.32 mg/L (DeLorenzo *et al.,* 2002; Naqvi and Vaishnavi, 1993). As with sediment/soil exposure, there is no agreement on the persistence in water. It has been suggested that endosulfan in water can persist anywhere from 3 days to 7 months (Siang *et al.,* 2007; Harris *et al.,* 2000; DeLorenzo *et al.,* 2002; Garret, 2004). Some of the variation can be attributed to water turbidity (helps volatilize α-endosulfan) (Siang *et al.,* 2007), pH (more acidic the pH the slower the degradation from 1 week – 5 months) (Harris *et al.,* 2002) and the level of dissolved oxygen which affect the rate of degradation (Harries *et al.,* 2000). Similar to sediment/soil, endosulfan in water undergoes oxidation and hydrolysis to form endosulfan sulfate. Along with this process, endosulfan in the water column can also undergo volatilization, and photolysis (EPA, 2002; Garret, 2004) converting endosulfan to its sulfate form. In Ontario, marshy wetlands adjacent to sprayed crops, or muck crop areas, could act as sinks for endosulfan. These areas have high potential for being eutrophic and could result in anaerobic sediments and seasonally low dissolved oxygen concentrations. This would result in longer persistence of endosulfan sulphate (Harris *et al.,* 2000).

2.2.7.3 Air

Please refer to section 2.2.6.2.

2.2.8 Bioaccumulation in Fish

Endosulfan persistence in the environment has been shown to be highly variable and has the potential to be highly bioaccumulative in fish. A study by the U.S. Environmental Protection Agency (2002) showed α and β-isomers to have an octanol-water partition coefficient (K_{ow}) of 55500 and 61400 respectively and bioconcentration

factors (BCF) of 2400 to 11000 times. K_{ow} values are representative of how well a compound can cross lipid barriers and enter fatty tissues inside and organism. These values are in agreement with another study by the U.S. Department of Health and Human Services (DHHS) (2002) which found similar results for both α and β-isomers and BCFs of less than 3000 times. Even with the moderate to high potential of endosulfan to bioaccumulate, both isomers tend to be quickly eliminated from the organism within a time span of 24 h – 2 weeks depending on the cleanliness of the water after exposure (EPA, 2002; DHHS, 2002; Naqvi and Vaishnavi, 1993; PMRA, 2007). If fish are continuously exposed to chronic levels of endosulfan it is possible that they will retain a small amount of endosulfan in their tissues (Henry and Kishimba, 2006) and that endosulfan may then travel up the food chain to higher trophic organisms.

2.2.9 Endosulfan Contamination and Mishaps

With the extensive use of endosulfan in Ontario and around the world, it is not surprising that incidences of spills and contamination have occurred. In 1969, 300-400 dace, white sucker, and rock bass were killed in the North Thames River, Ontario upon exposure to 0.096 - 0.26 mg/L endosulfan originating from off-target spraying of potato fields (Harris *et al.,* 2000). Another such accident occurred near Simcoe, Ontario in 1972 in which rainbow trout were killed in a pond exposed to endosulfan from a tobacco field (Harris *et al.,* 2000). India is another such country to have endosulfan contamination, with fish liver tissue concentrations of 61.92 µg/g wet weight reported (Amaraneni and Pillala, 2001), and fish found near cotton plantations found to have high residue levels (Naqvi and Vaishnavi, 1993). In both cases fish were exposed chronically from agricultural use of endosulfan.

Even though aquatic organisms tend to have a higher sensitivity to endosulfan than other organisms, endosulfan can still impact mammalian communities including humans. Those most at risk are those directly involved with its application and formulation (Paul and Balasubramaniam, 1997). In the USA there was one incident in which a farmer was killed, while in 1990 and 1993 there were 32 poisonings related to endosulfan in southern Sulawesi, Indonesia (EJF, 2002). Workers in India who have

applied endosulfan without protective equipment experienced: dyspnea, increased respiratory rates, tachycardia, bradycardia, and abdominal discomfort (DHHS, 2000). These are only a few of the documented cases in which contamination or injury has been linked to endosulfan.

2.2.10 Rainbow trout (*Oncorhynchus mykiss*) as a Test Species

Rainbow trout are ideal test species for acute and chronic tests (Tyler *et al.,* 2002) because they can be easily cultured in the lab, are not stressed easily during handling, are sensitive to a wide variety of toxicants, are more sensitive than other aquatic species, and can be obtained all year from commercial suppliers (EPA, 2002). Another feature that makes them ideal for testing of the proposed toxicants is that they are naturally found in the Oshawa Creek Watershed (originally were a stocked species) and surrounding watersheds which allows them to be a representative cold water species for the region (EPA, 2002; CLCOA, 2002).

2.3.0 Biomarkers and Histology

2.3.1 Vitellogenin

Vitellogenin (VTG) is a phospholipoprotein that is the precursor molecule to the synthesis of yolk during oocyte formation (Lal, 2007; Jensen and Ankley, 2006). Production of VTG occurs through the activation of estrogen receptors by 17-β estradiol, which in turn is controlled by the hypothalamic-pituitary-gonadal (HPG) axis (Jensen and Ankley, 2006). VTG analysis is utilized as a reliable biomarker for endocrine disruption and analytical methods have been developed for various species including; fathead minnows, rainbow trout, and crimson-spotted rainbowfish (*Melanotaenia fluviatilis*) (Eidem *et al.,* 2006; Jensen and Ankley, 2006; Xie *et al.,* 2005; Holdway *et al.,* 2007). VTG is reliable in showing estrogenic effects of xenobiotics with increased VTG production in male fish or anti-estrogenic effects due to a reduction in VTG production in female organisms (Jensen and Ankley, 2006; Xie *et al.,* 2005; Harris *et al.,* 2002). This

methodology is quite useful when looking at mixtures of xenobiotics, such as pulp mill effluents, urban waste waters, and agricultural runoff (Orrego *et al.,* 2009; Gagné and Blaise, 1998).

2.3.2 Ethoxyresorufin-O-deethylase

Ethoxyresorufin-O-deethylase (EROD) is a well established indirect biomarker (terrestrially and aquatically) for the induction of the mixed-function oxygenases (MFO), which play a crucial role in the degradation of xenobiotics (Kammann *et al.,* 2005). This biomarker has been established in approximately 150 fish species including; flounder (*Platichthys flesus*), dab (*Limanda limanda*), sand flathead (*Platycephalus bassensis*), and rainbow trout (*Oncorhynchus mykiss*) (Kammann *et al.,* 2005; Kirby *et al.,* 2007; Brumley *et al.,* 1995; Whyte *et al.,* 2000). One of the main proteins in the MFO system is the terminal component P-450 (CYP) 1A1 (Kirby *et al.,* 2007). P-450 system is thought to act by the binding of xenobiotics to the cytosolic aryl hydrocarbon receptor (AhR). Once bound, AhR binds to an aryl hydrocarbon nuclear translocation protein (ARNT). This binding initiates the transcription of several genes to produce the proteins P-450, which then leads to the detoxification of the xenobiotic and in rare cases a toxic response (**Figure 2.4**) (Whyte *et al.,* 2000). It is the enzymatic activity of P-450 that produces the oxidative deethylation of 7-ethoxyresorufin (7-ER) to resorufin (Petrulis *et al.,* 2000). This reaction is carried out in high enough concentrations of 7-ER to allow a fluorescence intensity that is proportional to the concentration of P-450 (Petrulis *et al.,* 2000).

2.3.3 Lactate Dehydrogenase

Lactate dehydrogenase (LDH) is a hydrogen transferring enzyme that catalyzes the last step in glycolysis (Kuznetsov and Gnaiger, 2006; Kurutaş *et al.,* 2006), by catalyzing the reversible oxidation of lactate ions to pyruvate ions with the accompanying reduction of NAD^+ to NADH (Dorey and Draves, 1998; Mishra and Shukla, 1997). CS

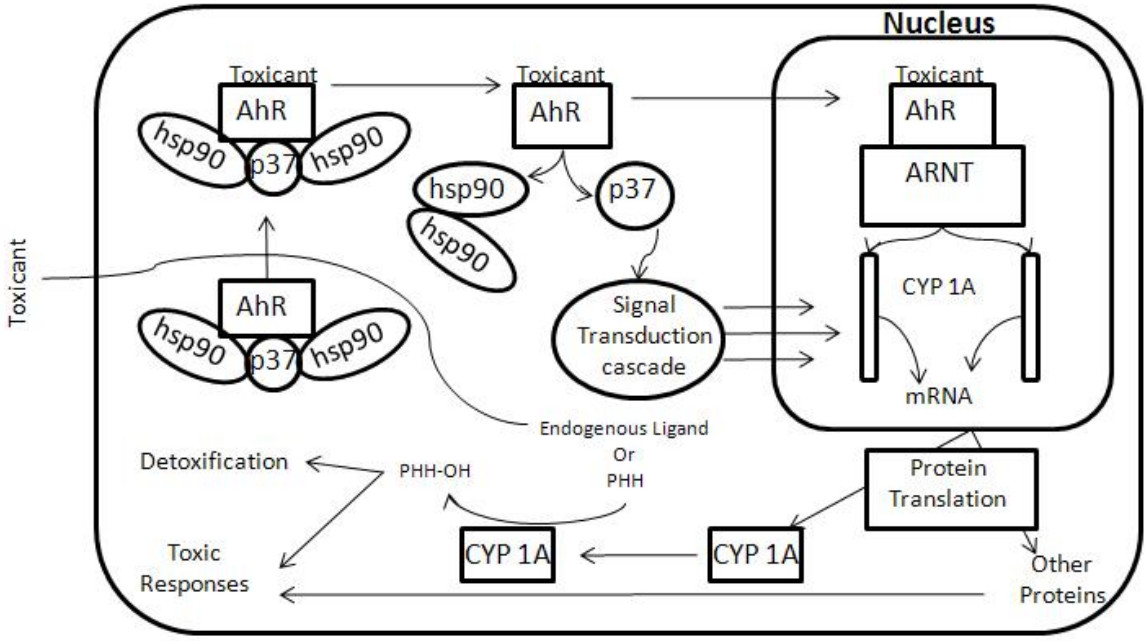

Figure 2.4: EROD mechanism of action. A toxicant binds to the AhR which is then transferred into the nucleus of a cell. There it initiates the synthesis of CYP1A RNA which is translated into CYP1A enzyme used to detoxify the causative chemical. It is the oxidation of 7-ER to resorufin by CYP1A that produces a colorimetric reaction which can then be used to indirectly quantify CYP1A activity.

and LDH are related functions where CS is a measure of aerobic metabolic capcity and LDH is a measure of anaerobic metabolic capcity; hence an increase in one may result in a decrease in the other (Konradt and Brunbek, 2001). Taylor *et al.,* (1973) found that the baseline levels for LDH for juvenile rainbow trout in liver ranged between 164-184 μmol/min/mg protein.

2.3.4 Citrate Synthase

Citrate synthase (CS) is a key enzyme in the citric acid cycle (Kreb cycle) and is found within the mitochondrial matrix (Kuznetsov *et al.,* 2006). It is nuclear encoded and thus synthesized outside the mictochindria by cytoplasmic ribosomes and then transported into the mitochondrial matrix (Kuznetsov *et al.,* 2006). CS catalyzes the reaction of aceytle CoA with oxaloacetate to form citrate, and regenerates coenzyme A (Acetyl-CoA + oxaloacetate + H_2O → citrate + CoA-SH) (Kuznetsov *et al.,* 2006).

2.3.5 Acetylcholine Esterase

Acetylcholine esterase (AChE) is an enzyme found on the post-synaptic membrane that hydrolyzes the neurotransmitter acetylcholine (ACh), which provides a pivotal role in the maintenance of normal nerve function (Sandhal and Jenkins, 2002; Liu *et al.,* 2007). ACh is synthesized in the pre-synaptic cleft (composed of acetyl-CoA and choline), and released into the synaptic cleft where it then reacts with receptors on the post-synaptic cleft which in turn causes a neurological response (Pope *et al.,* 2005; Malomouzh and Nikol'skii, 2007). In the case of AChE inhibition, there is a build up of ACh in the synaptic cleft, and continual firing at the receptors (overabundance of neurotransmitter) (Liu *et al.,* 2007). In muscle fibres this causes hyper activity, tetany, paralysis, convulsion and eventually death (Liu *et al.,* 2007; Siang *et al.,* 2007). This is important because these effects are commonly seen in fish following exposure to organophosphate and carbamate pesticides (Liu *et al.,* 2007; Cannard, 2006; Pope *et al.,*

2005). Thus AChE inhibition is an excellent biomarker for monitoring aquatic systems in agricultural areas.

2.3.6 Condition Factor

Condition factor is used as an indicator of fish well being and is a percentage of body weight compared to the fish length cubed (rainbow trout only). An increased condition factor is indicative of increased fish health (bigger/fatter), whereas a lower condition factor is indicative of decreased health (smaller, reduced fat stores) (Raymond *et al.,* 2001). Condition factor is dependent on the morphology of the fish species (Raymond *et al.,* 2001)

2.3.7 Hepatosomatic Index

The hepatosomatic index (HSI) is the percentage of body weight attributable to the liver and can be used as an indicator of fat deposits and protein induction (Raymond *et al.,* 2001). HSI is also a crude indicator of liver activity with a larger liver in proportion to body weight indicating increased liver activity, possibly from toxicant removal. Similar to condition factor, this index is variable and is dependent on the fish species being monitored.

2.3.8 Gonadosomatic Index

Gonadosomatic index (GSI) is the percentage of body weight attributable to the gonads and can be used as an indicator of fish sexual maturation (Ma *et al.,* 2005). Increased GSI is indicative of increased gonad size and fish sexual maturation, where decreased GSI could be indicative of inhibited sexual function or maturation (Ma *et al.,* 2005).

2.4.0 Injections and Field Tools

2.4.1 Intra-peritoneal Injection

Intra-peritoneal (i.p.) injections are one of the most commonly used methods for administering fish vaccines and other treatments in various vertebrates (Nakanishi *et al.,* 2002; Wilson and Holberton, 2001). This method is an excellent way to introduce endocrine disrupting chemicals into test organisms (Wilson and Holberton, 2001). An advantage to this methodology is that it bypasses the natural skin and mucus barriers of fish leading to a faster uptake of the desired treatment (Itano *et al.,* 2006). It also allows for a specific and known dose to be administered compared to waterborne exposures where there is no control on how much toxicant is taken up. Another advantage is that this method has been successfully used in numerous fish species including rainbow trout, sea bream, and channel catfish to name a few (Snow *et al.,* 2001; della Rocca *et al.,* 2004; Xu *et al.,* 2004). Negative aspects of using i.p. injections are increased stress of the fish, reduced environmental relevance, higher labour costs compared to water exposure, and increased health risks to the researchers (Nakanishi *et al.,* 2002). Injecting fish has resulted in numerous cases of slipped needles and accidental injections into the hands of the administrators resulting in various health issues (O'Neill *et al.,* 2005). Overall, i.p. injections are an effective method to rapidly introduce a vaccine or toxicant into a fish under similar metabolic conditions compared to waterborne and food borne exposures (Tencella *et al.,* 1994; Sherry *et al.,* 1999).

2.4.2 Semi-permeable Membrane Devices

Semi-permeable membrane devices (SPMDs) have been utilized in the field to mimic environmental exposure over time and concentrate various chemicals, predominantly polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and organochlorine pesticides (Verwij *et al.,* 2004; Zimmerman *et al.,* 2000). The general design of SPMDs includes a thin film of highly refined lipid (triolein) sealed inside a thin walled polyethylene tube. Lipophilic compounds then permeate the polyethylene

membrane and are partitioned in the lipid and retained until the SPMD is analyzed (Prest *et al.,* 1995; Ellis *et al.,* 1995; Stuer-Lauridsen and Kjølholt, 2000). The molecular pore size of the polyethylene tubing mimics those found in nature and the small pore size provides minimal contribution of particle bound contaminants (Sabaliūnas and Södergren, 1997). Along with their ability to mimic biological processes, SPMDs contain performance reference compounds which allow for site specific environmental factors to be included in the analysis (USGS, 2004). SPMDs have a wide versatility for accumulating various families of lipophilic chemicals and they are an essential tool for monitoring aquatic contaminants where a wide variety of possible contaminants are potentially present.

2.4.3 Thermogravimetric Analysis

Thermogravimetric analysis (TGA) is the characterization of organic matter through the heating and combustion of a given sample (Payá *et al.,* 1998; Grisi *et al.,* 1998). Two predominant analytical methods have been developed that both utilize muffle furnaces. In the first method, the furnace is hooked up to a computer and uses sophisticated software by which to program specific temperature profiles. The second method is a straight weight change determination after a specified amount of time (Grisi *et al.,* 1998). In aquatic and terrestrial mixtures, organic matter is typically composed of carbohydrates, lipids, proteins, polyphenols, and complex macromolecular humic substances (Kristensen, 1990). It is the temperature at which these materials combust that identifies the organic matter composition and total organic matter content of a soil, sediment, or other biological matrix (e.g. sewage sludge) (Grisi *et al.,* 1998; Folgueras *et al.,* 2003; Boyle 2004).

2.4.4 Caging

Caging fish is a methodology used to monitor aquatic environments that have been exposed to chemical contaminants purposely or accidentally (Oikari, 2006). Unfortunately, cage designs and techniques have not been harmonized nationally or internationally (Oikari, 2006). Caging is advantageous because it allows the transplantation of "clean" fish into a contaminated area to monitor various biomarkers in the fish such as EROD and AChE (Fenet *et al.,* 1998).

When designing a caged experiment, the type of fish must be considered because some species stress easily, do not do well when placed in larger populations, or are cannibalistic (Oikari, 2006). Overall, two fish species that have proven to work well in caged experiments are rainbow trout and fathead minnows since they are responsive to chemical contaminants (more sensitive than other native species), and can be easily handled without stressing the fish (Oikari, 2006; Hanson *et al.,* 2006).

Since there is large variability with cage designs, locations, and environmental factors, caged experiments are interpreted cautiously because it is hard to identify a single contaminant and variability between locations could significantly skew the results (Oikari, 2006).

3. Aims of the study

The primary aim of the different sections of this study was to determine the chronic effects of a single i.p. injection of endosulfan on a population of juvenile rainbow trout (*Oncorhynchus mykiss*) and to take 30 day field observations of Oshawa Creek at two times of year (spring and fall) to determine any possible effects of endosulfan, location and season on caged rainbow trout. Due to the lack of conclusive evidence from the surrounding agricultural sector for the use of endosulfan within the watershed, the field and the laboratory experiments became separate entities.

It was hypothesized that a single i.p injection of endosulfan will cause a noticeable effect in liver EROD, CS, LDH, plasma VTG, HSI, GSI and condition factor. Alternatively, endosulfan will not affect the various biomarkers in rainbow trout after a single i.p. injection of endosulfan.

It was also hypothesized that potential contaminants in various regions of Oshawa Creek would have a negative effect in rainbow trout placed along its course for 30 days. Alternatively, caging of fish within Oshawa Creek would have no effect on liver EROD, CS, LDH, blood plasma VTG, HSI, GSI, brain AChE and condition factor in the caged trout.

4. Materials and Methods

4.1.0 Chemicals and Reagents

All chemicals and reagents were obtained from Sigma Aldrich (Oakville, Ontario Canada) and Fisher Scientific (112 Colonnade Road, Ottawa, Ontario, K2E 7L6) except for polyclonal antibody (Rabbit anti-Sea Bream) for trout vitellogenin which was acquired from Biosense Laboratory (Thormøhlensgt, 55 Bergen, N-5008, Norway).

4.2.0 Fish Sampling

All trout were anaesthetized with MS-222 (90 mg/L) in 10 L of water until there was no response from outside stimulus. They were then placed ventral side up and blood was removed from the caudal vein with a 22G1 blood sampling needle and a 4-6 ml vaccutainer. The trout were then opened longitudinally by inserting scissors in the anus and cutting towards the head, ending just after the operculum. Tissues excised include liver, kidney, 2nd gill arch, gonads, fat, muscle and brain. All tissues were placed in 2 ml cryo-tubes and stored in a -80 ^{o}C freezer, with the exception of the gill arches and gonads which were stored in 4 % formalin in 1.7-2 ml micro-centrifuge tubes. Blood containing vaccutainers were spun at 4000 rpm at 4 ^{o}C for 10 minutes, supernatant removed and placed in 1.7 ml micro-centrifuge tubes. The blood plasma was then stored in a -80 ^{o}C freezer.

4.3.0 Tissue Analysis

4.3.1 Bradford Protein Analysis

Bradford protein analysis was performed on liver, brains, and blood plasma. The technique used was modified from a cuvette technique by Bradford (1976). Samples were diluted in Milli-Q water (1/5 liver, 1/40 brain, and 1/50 blood plasma) and plated in triplicate on a 96 well UV micro-plate. 225 µl of Bradford reagent (Bioshop Inc.) was added to each well except blanks and incubated for 45 minutes. Absorbance was read in a (Bio-Tek Synergry HT micro plate reader) plate reader at 595 nm.

4.3.2 7-Ethoxyresorufin-O-deethylase

Livers were sectioned with 50 mg of liver being added to 250 ul of HEPES grinding buffer and put into a 2 ml micro-centrifuge tube. They were then homogenized at level 3 for 5-8 s using an Ika: T-25 basic Ultra-Turrax homogenizer. Liver homogenate was then centrifuged at 9000 g for 20 minutes at 2 ºC. The supernatant was further collected in 1.5 ml micro-centrifuge tubes, thus containing within the supernatant the S9 fraction of the liver. Supernatants were stored at -80 ºC until further analysis.

Hepes grinding buffer was prepared by dissolving 5.592 g potassium chloride and 2.603 g HEPES in 500 ml of Milli-Q water and adjusted to pH 7.5, then stored at 4 ^{o}C.

Hepes buffer was prepared by dissolving 13.015 g Hepes in 500 ml of Milli-Q water and adjusted to pH 7.8 and also stored at 4 ^{o}C.

Next, 0.022 mg of 7-ethoxyresorufin was dissolved in 1 ml DMSO (dimethyl sulfoxide). It was then checked for absorbance (461.5 nm) to ensure that it read between 1.60 and 1.70 absorbance units. A volume of 550 µl was required per plate. It was then stored at -20 ^{o}C wrapped in tinfoil.

NADPH was prepared at the moment of use and required 10 mg of NADPH per 1 ml of Milli-Q water per plate.

Resorufin standards were prepared by creating a super stock solution which was diluted to produce working solutions. Super stock solutions consisted of 5.0 mg resorufin dissolved in 1 ml DMSO (this may take several days). Once dissolved the super stock

was wrapped in tin foil and stored at -20 ^{o}C. The working solution was diluted from the super stock; by diluting 1 ml super stock in 9 ml DMSO. This was stored in the same manner as the super stock. The working solution was used to create the standard curve: 10 µl of working solution was diluted in 4990 µl of HEPES grinding buffer and aliquoted into 5 test tubes containing 0, 200, 400, 600, 800 µl and 1000, 800, 600, 400, 200 µl of working solution and HEPES grinding buffer respectively.

Once the livers were homogenized, 50 µl of standard were added to wells B-F in triplicate in a 96 well, non-binding flat bottomed, black polystyrene microplate (well A was used for a blank). 50 µl of homogenized liver S9 fraction was then added to the remainder of the plate in triplicate. 550 µl of 7-ethoxyresorufin was combined to 4550 µl of HEPES buffer and then 50 µl was added to every well of the plate except the blanks. The plate was then incubated in the dark at room temperature for 10 minutes. Once incubation was completed 10 µl of NADPH was added to every well except the blanks to start the enzymatic reaction. Once the NADPH was added to the plate, the plate was immediately read. The KC4 EROD protocol was set with an excitation filter of 530 nm (30 nm bandwidth), an emission filter of 590 nm (35 nm bandwidth) and a sensitivity set to wells F1-F3. Data collected consisted of fluorescent units per minute for liver samples and mean slope for the resorufin calibration curve.

4.3.3 Enzyme Linked Immunosorbent Assay (ELISA) for fish Vitellogenin

The ELISA method to test for fish VTG was based on those described by Orrego *et al.,* (2009). 96 well flat bottomed, half area, high binding, polystyrene plates from Corning were utilized as they effectively allow for half the reagents to be used. Blood plasma was diluted in coating buffer (1.59 g sodium carbonate and 2.93 g of sodium bicarbonate dissolved in 1 L Milli-Q water and adjusted to a pH of 9.3) to a final concentration of 50 µg/ml (final protein dilutions were determined from a previous Bradford assay). 50 µl of sample was transferred to the plate in triplicate with 100 µl of coating buffer being added in triplicate to the first wells of the plate (blank). The plate was placed in a plastic container with a moist Kimwipe and left for 12 hours at 4 ^{o}C.

After the incubation period the plate was washed three times with 0.05 % TWEEN-20 in phosphate buffered saline (PBS) (TPBS) (PBS: 1.15 g of Na_2HPO_4, 0.2 g KH_2PO_4, 8.0 g NaCl, and 0.2 g KCl dissolved in 1 L Milli-Q water and adjusted to pH 7.3. TPBS: 100 ml PBS with 500 µl Tween-20 made up to a final volume of 1 L with Milli-Q water). During the third wash the plate was allowed to sit for 5 minutes before removing the TPBS.

The plate was then blocked by adding 100 µl of blocking solution (2 % Bovine Serum Albumin (BSA) in PBS) to each well of the plate minus the blank. The plate was then placed back in the plastic container and incubated at room temperature for 1 h followed by another washing sequence.

Polyclonal antibody, rabbit anti-sea-bream was used as the primary antibody. Primary antibody was used at a concentration of 1/1000 in 1 % BSA in PBS. This was added at a volume of 50 µl per well except the blank and incubated for 24 hours at 4 ^{o}C. This was followed by another wash sequence.

Peroxidase sheep anti- IgG rabbit was used as the secondary antibody at a concentration of 1/5000 in 1 % BSA in PBS. This was added to the plate at 50 µl to all sample wells. The plate was then allowed to incubate for 1 h at room temperature followed by another washing sequence.

Finally the plate was developed by adding 50 µl of developing solution (1 o-Phenylenediamine dihydrochloride (OPD) tablet in 24 ml Solution A (2.1014 g Citric acid dissolved in 100 ml of Milli-Q water), 25 ml Solution B (3.5598 g $Na_2HPO_4 \cdot 2H_2O$ dissolved in 100 ml Milli-Q water), and 15 µl 30 % hydrogen peroxide) to each well and then incubated at 37 ^{o}C for 30 minutes. The reaction was stopped by the addition of 50 µl of 1.8 M H_2SO_4 to all sample wells. The plate was then read at 492 nm on the absorbance setting. VTG concentrations were then determined by comparison to the standard curve. The standard curve was performed in triplicate and used concentrations of 25, 50, 100, 250, 500, 750, and 100 ng/ml of VTG.

4.3.4 Liver Lactate Dehydrogenase

Trout liver samples were homogenized using an Ika: T-25 basic Ultra-Turrax homogenizer on level 3 for 30 s in 9 volumes of imidazole buffer (3.4 g imidazole dissolved in 1 L of Milli-Q water, adjusted to pH 7.6) and centrifuged at 2300 g for 10 minutes at 4 ^{o}C. The supernatants were collected and stored at -80 ^{o}C until further analysis.

Samples were thawed and 25 µl were transferred in triplicate to a 96 well UV micro-plate and incubated at room temperature for 10 minutes. During the incubation time the dosing solution was mixed (25 ml of NADH (4.729 mg of NADH dissolved in 25 ml imidazole buffer)) and 5 ml of sodium pyruvate (3.668 mg sodium pyruvate dissolved in 5 ml of imidazole buffer) and incubated at 25 ^{o}C. After incubation 225 µl of dosing solution was added to each well to initiate the reaction. The plate was read to measure the change in absorbance at 340 nm at 30 second intervals for 3 minutes at 25 ^{o}C.

4.3.5 Liver Citrate Synthase

Livers were homogenized and supernatants collected in the same fashion as described for LDH. Samples were transferred to sample and control wells in duplicate and 150 µl of Tris buffer (121.14 g Tris (Tris-(hydroxymethyl)-aminomethan) in 1 L of Milli-Q water and adjusted to pH 8.1) to sample wells and 175 µl to control wells. The plate was incubated at room temperature for 5 minutes and then the addition of 25 µl of (5,5'-dithiobis(2-nitrobenzoate)) (DTNB) (1.1889 mg dissolved in 3 ml of Tris buffer) was added to all wells. The plate was then incubated for another 5 minutes. Following incubation, 25 µl of 3 Mm acetyle-CoA was added to every well and the plate incubated again for 10 minutes at room temperature. At this point 25 µl of oxaloacetate (0.297 mg dissolved in 1.5 ml of Tris buffer) was added to all the sample wells to initiate the reaction. The absorbance of the samples was read at 412 nm at 30 second intervals for 5 minutes.

4.3.6 Condition Factor

Condition factor was calculated by: $(fish\ weight\ \div\ fish\ standard\ length^3)\ \times 100$.

4.3.7 Hepatosomatic Index

Hepatosomatic index was calculated by: $(liver\ weight\ \times 100)\ \div\ fish\ weight$.

4.3.8 Gonadosomatic Index

Gonadosomatic index was calculated by: $(gonad\ weight\ \times 100)\ \div\ fish\ weight$.

4.4.0 Statistical Analysis

All data was tested for normality and homogeneity of variance with a Shapiro-Wilks test and Brown-Forsyth test respectively. Laboratory exposure 2, EROD was the only test to fail the Brown-Forsyth test and was transformed by an inverse regression. All data was tested at $\alpha = 0.1$ to decrease the chances of Type II error and by 2-way factorial ANOVA to test if gender had any significant effects, if so those tissue analysis were split and analyzed by gender. Both laboratory exposures were analyzed by 2-way factorial ANOVA. Both field exposures were analyzed by 1-way ANOVA. All abiotic factors were also analyzed by 1-way ANOVA. Fishers LSD test was used when significance was indicated from the fore mentioned ANOVAs.

5. Laboratory Endosulfan Exposure 1

5.1.0 Introduction

Endosulfan a non-systemic organochlorine pesticide used by many countries around the world including Canada and the United States (Harris *et al.,* 2000; Siang *et al.,* 2007). In Ontario, from Durham region and North to Georgian Bay endosulfan has the highest use for agricultural purposes (Harris *et al.,* 2000) but, in recent years it use has been in decline (Tuduri *et al.,* 2006). Due to its prevalent use, toxicologically relevant concentrations in aquatic ecosystems (Bernabo *et al.,* 2008) and high toxicity to various aquatic species (LC_{50}: 1.7 µg/L for rainbow trout and bluegill sunfish (*Lepomis macrochirus*), 1.5 µg/L for fathead minnows (*Pimephales promelas*), and 0.1 µg/L for striped bass (*Mornone saxatillis*) (Capkin *et al.,* 2006; EPA, 2002)). Endosulfan has the potential to greatly disrupt aquatic populations and ecosystems and has been shown to have sub-chronic effects and be an endocrine disrupting compound causing feminization of Japanese medaka and decreased testicular testosterone in rats (Gormley and Teather, 2003; DHHS, 2000).

In rainbow trout (*Oncorhynchus mykiss*) endosulfan has caused a wide range of effects including: hyperactivity, convulsions, paralysis, erratic behaviour, and eventually death through water borne and feed borne exposures (Harris *et al.,* 2000; Broomhall S., 2002; Brunelli *et al.,* 2009; Naqvi and Vaishnavi, 1993; Yu, 2008). Very little work has been done with respect to intra-peritoneal injections (i.p.) of endosulfan and its subsequent effects on metabolic enzymes and the reproductive biomarker vitellogenin. In one study involving rainbow trout, i.p. injections, and monitoring of vitellogenin found no induction of vitellogenin at 9 days after injection (Andersen *et al.,* 1997). However, vitellogenin induction may have occurred and returned to base levels before vitellogenin was analyzed (Orrego *et al.,* 2009).

The aim of this study was to investigate the effects of endosulfan on various metabolic enzymes and its potential ability to cause feminization in rainbow trout via a

single i.p. injection at four different concentrations. Biomarkers including liver citrate synthase, lactate dehydrogenase, cytochrome P450 activity and blood plasma vitellogenin induction were investigated and correlated to dose.

5.2.0 Materials and Methods

5.2.1 Fish

Rainbow trout (*Oncorhynchus mykiss*) were obtained on May 7th, 2008 from Rainbow Springs Trout Farm in Thamesford, ON. The batch contained 247 trout (197 ± 64 g). They were held in 1500 L tanks and maintained at 11.8 ± 0.23 ^{o}C with a dissolved oxygen content of 9.5 ± 0.19 ppm and acclimated in lab for two weeks before use.

5.2.2 Stock and Working Solutions

5.2.2.1 Endosulfan Stock Solution

Endosulfan stock solution was created by weighing 70 mg of 2:1, α:β-endosulfan (analytical standard) into a 2 ml micro-centrifuge tube on a micro-balance. In the fume hood 1 ml of acetone was used to dissolve the endosulfan. This was then added to 9 ml of acetone in a 10 ml brown injection bottle. Contents were mixed on a vortex for ~10 s. Final stock solution created was 7 mg/ml and stored in a plastic box in the -20 ^{o}C freezer until needed.

5.2.2.2 Endosulfan Working Solutions

From the endosulfan stock solution, 4 working solutions were produced (0.1, 0.32, 1, and 3.2 mg/ml). In each working solution the process was identical except for the amount of corn oil or endosulfan used. Briefly for the 0.1 mg/ml working solution; 0.143 µl of stock solution was combined with 9.857 ml of corn oil in a 10 ml brown injection

bottle. The bottle was then capped with a rubber septum and sealed shut by crimping a metal ring around the rubber septum and the top of the bottle. The solution was stored alongside the stock solution in the -20 ^{o}C freezer. For the 0.32, 1, and 3.2 mg/ml working solutions; 0.457, 1.429, and 4.571 ml endosulfan stock solution were combined with 9.543, 8.571, and 5.429 ml of corn oil respectively. All solutions were thawed for 1 h before use and mixed with a vortex for ~20 s.

5.2.2.3 17-β-estradiol

Estradiol solution was created by weighing out 50 µg of 17-β-estradiol (≥ 98 %) into a 2 ml micro-centrifuge tube on the micro-balance. To this 1ml of acetone was used to dissolve the 17-β-estradiol by mixing it on a vortex for ~10 s. This solution was then added to 9 ml of corn oil in a 10 ml brown injection bottle and mixed for another 10 s and sealed as previously mentioned. Again this solution was stored alongside the endosulfan stock solution in the -20 ^{o}C freezer until needed.

5.2.2 Injections

Injections commenced on August 25th and 26th, 2008. Over the 2 days 120 fish were injected and an additional 20 were measured for experimental controls. Of the injected fish; 20 were carrier controls (corn oil), 20 were positive controls (17-β-estradiol), and 80 were injected with four endosulfan treatments (0.1, 0.32, 1, and 3.2 mg/kg endosulfan (20 fish per treatment)). Injections were carried out by anesthetising the trout with MS-222 (90 mg/L) in 10 L of water. Once the fish were non-responsive to outside stimulus they were transported to a scale and weighed to the 100th of a gram and measured for total, fork, and standard lengths. The trout were then placed ventral side up in a plastic container which allowed for constant water flow and fresh water to be passed over the gills. They were then injected using a Socorex© 187 pistol grip, vial feeding syringe and rotated, leaving the dorsal side up. Trout were then tagged with an Avery Dennison© tagging gun and coloured t-bar anchor tags (colour of tag depended on

treatment). Tags were placed in the muscle, port side just before the dorsal fin. Once injected and tagged, trout were placed in a recovery bucket with an air stone. Once recovered they were placed into one of two 1500 L tanks (10 fish from each treatment per tank).

Fish were sampled on 0, 2, 4, 8, 16, and 28 days after injection and analyzed as per 4.2.0 – 4.5.0.

5.3.0 Results

In all biomarkers tested, the only one significantly affected by the sex of the fish was liver LDH, all other data was pooled for gender and analyzed accordingly.

Since trout were not tagged individually and separated by tank when sampling, only final weights and standard lengths for each treatment over 28 days are shown in **Table 5.1** and **Table 5.2**.

It should be noted that there was mortality in the highest treatments (1.0 and 3.2 mg/kg endosulfan) before their timed sampling dates. There was mortality 1 day before the first sampling period in which four trout in the 3.2 mg/kg endosulfan treatment died. Before the second sampling period four trout in 3.2 mg/kg endosulfan died again along with 2 from 1.0 mg/kg endosulfan treatment. Lastly before the third sampling period there was a loss of 2 trout from 3.2 mg/kg endosulfan treatment.

Exposure of rainbow trout to endosulfan by single i.p. injection had no significant effects of GSI, HSI, condition factor, and standard length. However, exposure did have an effect on liver EROD, CS, female LDH, and blood plasma VTG (**Figures 5.3.1 – 5.3.6**).

Exposure to 0.32 mg/kg endosulfan caused a 2-fold decrease in liver EROD activity in fish sampled on day 2 compared to fish sampled at day 16 (**Figure 5.1** and **Figure 5.2**). Fish exposed to 0.1 mg/kg endosulfan at day 28 had significantly lower EROD than fish sampled on day 16. Fish injected with 1.0 mg/kg endosulfan sampled on

day 16 were significantly lower compared to corn oil controls sampled on days 2, 8, 16. Fish injected with the 0.32 mg/kg endosulfan had decreased EROD activity at days 2 and 4 compared to fish sampled on day 28 (1.5 and 1.0-fold respectively). Fish exposed to 3.2 mg/kg endosulfan and sampled on day 4 had a 3.0, 2.5, 4.0, and 2.0-fold increase in EROD activity compared to fish exposed to 3.2 mg/kg endosulfan sampled on days 2, 8, 16, and 28, respectively. EROD activity was also significantly higher than the corn oil controls on all days.

The majority of liver EROD activities across all endosulfan treatments were below time zero fish except for trout sampled at day 4 exposed to 3.2 mg/kg endosulfan. Fish EROD at time zero was significantly induced compared with fish exposed to 0.1 mg/kg endosulfan (fish sampled on days 2, 4, 8, 28), fish exposed to 0.32 mg/kg endosulfan across all sample days, fish exposed to 1.0 mg/kg endosulfan (sample on days 8-16), and fish exposed to 3.2 mg/kg endosulfan (sampled on days 2, 8, 16, 28).

Trout injected with higher endosulfan treatments (1.0 and 3.2 mg/kg endosulfan) had a greater induction of EROD activity compared to fish exposed to the lower treatments (0.1 and 0.32 mg/kg endosulfan) but, were declining as EROD activity in trout exposed to the lower doses started to increase. Trout exposed to 1.0 mg/kg endosulfan had a 2.0-fold increase in EROD activity compared to fish exposed to 0.32 mg/kg endosulfan sampled on day 2 and fish exposed to 3.2 mg/kg endosulfan was significantly higher than all other endosulfan treatments sampled on day 4.

EROD activity in fish exposed to 17-β estradiol was 2.0 – fold lower compared to corn oil controls sampled on day 8 but, recovered when compared to fish in the same treatment sampled on day 16 (increase of 2.0 – fold).

Liver citrate synthase activity was reduced by endosulfan exposure with LDH of fish exposed to 0.1 mg/kg endosulfan sampled on day 2 and 4 inhibited by approximately 3-fold compared to carrier controls fish. Fish injected with 0.1 mg/kg endosulfan experienced a recovery in CS activity at day 8 with a 4 – fold increase compared to trout in the same treatment sampled on day 4 (**Figure 5.3** and **Figure 5.4**).

Table 5.1: Weight of time zero fish (TZ), control rainbow trout (EC) and fish exposed to, corn oil (CO), 5 mg/kg 17-β estradiol (E2), and endosulfan (0.1, 0.32, 1 and 3.2mg/kg) over 28 days. Values are given as means ± standard deviation.

	TZ	EC	CO	17-B
Time zero	157.91 ± 20.30			
Day 2		183.84 ± 24.54	188.51 ± 37.71	141.29 ± 29.11
Day 4		211.99 ± 39.37	177.46 ± 53.54	132.49 ± 34.04
Day 8		194.81 ± 74.13	249.97 ± 23.10	165.00 ± 20.98
Day 16		242.67 ± 72.26	223.29 ± 39.28	233.39 ± 29.34
Day 28		191.53 ± 65.75	242.07 ± 31.21	248.83 ± 77.64

	0.1	0.32	1	3.2
Day 2	190.49 ± 60.15	198.67 ± 46.77	223.26 ± 30.07	195.04 ± 65.08
Day 4	161.95 ± 21.71	233.75 ± 90.27	127.68 ± 93.48	151.09 ± 36.32
Day 8	188.56 ± 59.90	234.51 ± 71.68	226.18 ± 47.60	147.10 ± 42.06
Day 16	191.92 ± 57.42	255.72 ± 24.20	256.07 ± 92.42	223.50 ± 48.12
Day 28	272.15 ± 84.73	174.67 ± 94.32	166.66 ± 72.82	209.49 ± 67.30

Table 5.2: Standard length of time zero fish (TZ), control rainbow trout (EC) and fish exposed to corn oil (CO), 5 mg/kg 17-β estradiol (E2), and endosulfan (0.1, 0.32, 1 and 3.2 mg/kg) over 28 days. Values are given as means ± standard deviation.

	TZ	EC	CO	17-B
Time zero	23.93 ± 1.34			
Day 2		24.60 ± 1.27	24.00 ± 1.58	22.50 ± 1.58
Day 4		24.98 ± 1.44	26.18 ± 1.41	22.53 ± 2.19
Day 8		24.38 ± 2.43	26.68 ± 1.16	23.75 ± 1.55
Day 16		25.25 ± 3.52	25.70 ± 1.67	26.00 ± 1.41
Day 28		23.55 ± 2.58	25.20 ± 1.12	25.73 ± 2.49

	0.1	0.32	1	3.2
Day 2	25.23 ± 1.54	25.45 ± 2.50	25.73 ± 1.07	24.00 ± 2.31
Day 4	23.13 ± 0.85	25.83 ± 3.29	21.35 ± 4.79	23.45 ± 2.49
Day 8	24.18 ± 2.67	26.38 ± 2.04	26.15 ± 1.27	23.08 ± 1.85
Day 16	24.38 ± 1.60	26.55 ± 1.32	26.3 ± 2.89	25.78 ± 1.00
Day 28	26.60 ± 1.35	23.23 ± 3.35	25.9 ± 2.87	24.88 ± 2.31

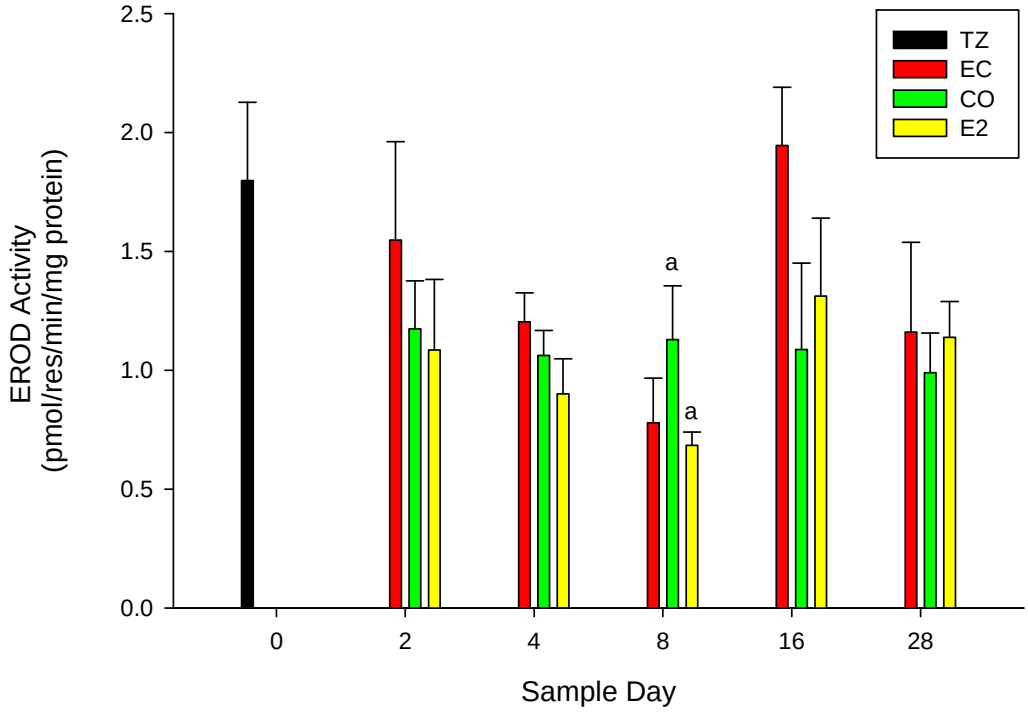

Figure 5.1: Liver EROD activity of time zero fish (TZ), control trout (EC) exposed to nothing, corn oil (CO) and 17-β estradiol (E2). Values are given as means ± standard error. Note bars follow the order as appearing in the legend. a denotes significance between 17-β estradiol and corn oil.

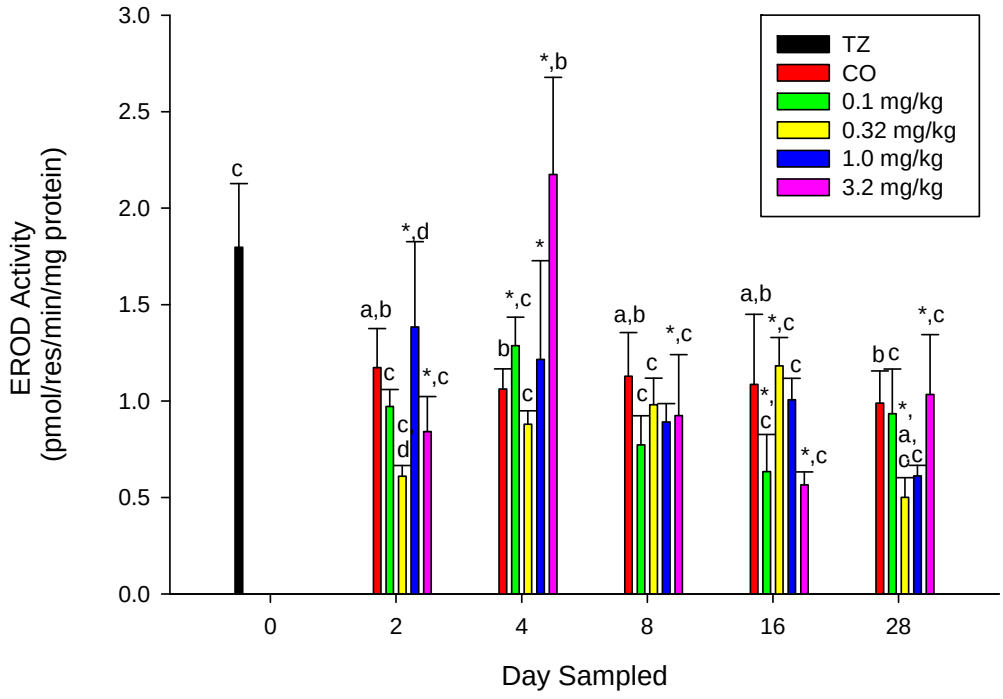

Figure 5.2: Liver EROD activity of time zero fish (TZ) and rainbow trout exposed to endosulfan (0.1, 0.32, 1 and 3.2 mg/kg). Data is presented as means ± standard error. Note bars follow the order as appearing in the legend. * denotes significance between matching treatments across time, a denotes significance compared to 0.32 mg/kg at day 28, b denotes significance compared to 3.2 mg/kg, day 4, d denotes significance between 0.32 mg/kg and 1.0 mg/kg, day 2, and c denotes significance compared to time zero.

Similar effects were seen for fish exposed to 1.0 mg/kg endosulfan but, a recovery in CS activity did not appear until day 28. CS activity in trout exposed to 0.32 mg/kg endosulfan was only inhibited at day 16 compared to corn oil controls. A decrease in CS activity of 0.21 ± 0.09 μmol/min/mg protein was experienced by fish exposed to 0.32 mg/kg endosulfan sampled on day 16 compared to fish sampled on day 8. Fish exposed to 3.2 mg/kg endosulfan sampled on day 8 and 16 had 2.5 – 3 fold of 0.25 ± 0.11 and 0.24 ± 0.12 μmol/min/mg protein inhibited CS activity, respectively, compared to similarly treated fish sampled on days 2 and 4, and corn oil controls. Fish exposed to 3.2 mg/kg endosulfan had a partial recovery in CS activity day 28. It should be noted that corn oil control fish had a lowered CS activity over time and controls sampled on day 28 had significantly lower CS activity than controls exposed at all other sampling periods.

Exposure to 17- β estradiol caused CS activity inhibition in livers of fish sampled on day 4 onwards and was significantly lower than the corn oil control trout sampled on days 4 through 16.

There was a significant difference in liver LDH activity between sexes in both experiments. In the first experiment, there was no significant difference in male liver LDH for any treatments at any day sampled but there were a significant treatment differences in LDH activity for female (**Figure 5.5**). Fish exposed to 0.1 mg/kg endosulfan had induced LDH activity at days 8 and 16 relative to the controls. At day 28 LDH activity in 0.1 mg/kg injected trout returned back to control and 2 day levels. LDH activities at days 8 and 16 in 0.1 mg/kg injected fish were greater than respective controls. Trout injected with 0.32 mg/kg endosulfan had significantly induced LDH activity after 2 days, but significantly decreased back to control levels on the sollowing sampling days. Female trout injected with 1.0 mg/kg endosulfan had a significant induction in LDH activity at day 28 relative to controls. On all of the other sampling days female LDH in all endosulfan treatments was lower than corn oil controls and not different from each other. Fish dosed with 0.32 and 1.0 mg/kg endosulfan had the highest recorded overall LDH activity of 0.67 and 0.69 ± 0.02 μmol/min/mg protein respectively.

Time zero fish liver LDH activity was higher than most treatments across all days except compared to trout injected with 0.32 mg/kg endosulfan sampled on day 2 and fish injected with 3.2 mg/kg endosulfan sampled on day 28.

There were no differences in plasma VTG between male and female trout exposed to endosulfan and 17-β estradiol. All concentrations were about 40 to 60 – fold increased compared to the corn oil controls on sample day 2 except for 0.32 mg/kg endosulfan injected fish (**Figure 5.6**). After day 2, all VTG activity declined to below 2 – fold higher than corn oil controls for all days except for day 8. Day 8 was significantly induced (4 to 10 – fold) compared to corn oil controls but was not different compared to day 4 continuing to show the decline in VTG activity for the duration of endosulfan exposure.

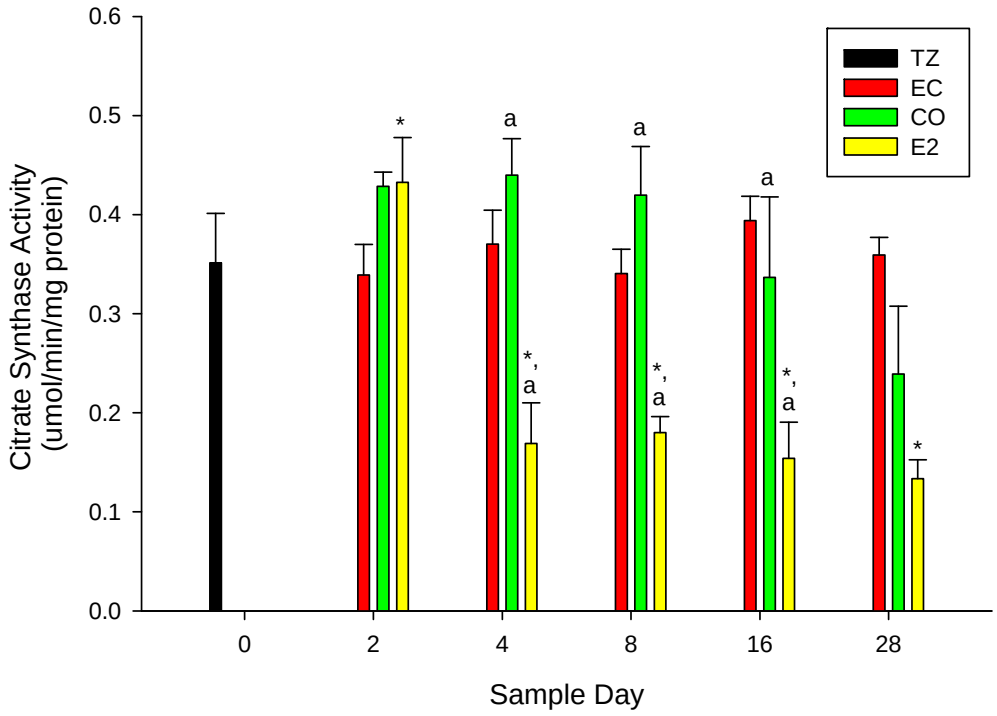

Figure 5.3: Liver CS activity of time zero fish (TZ), control trout (EC) exposed to nothing, corn oil (CO) and 17-β estradiol (E2). Values given as means ± standard error. Note bars follow the order as appearing in the legend. * denotes significance between 17-β estradiol compared to day 2 and a signifies difference compared to corn oil.

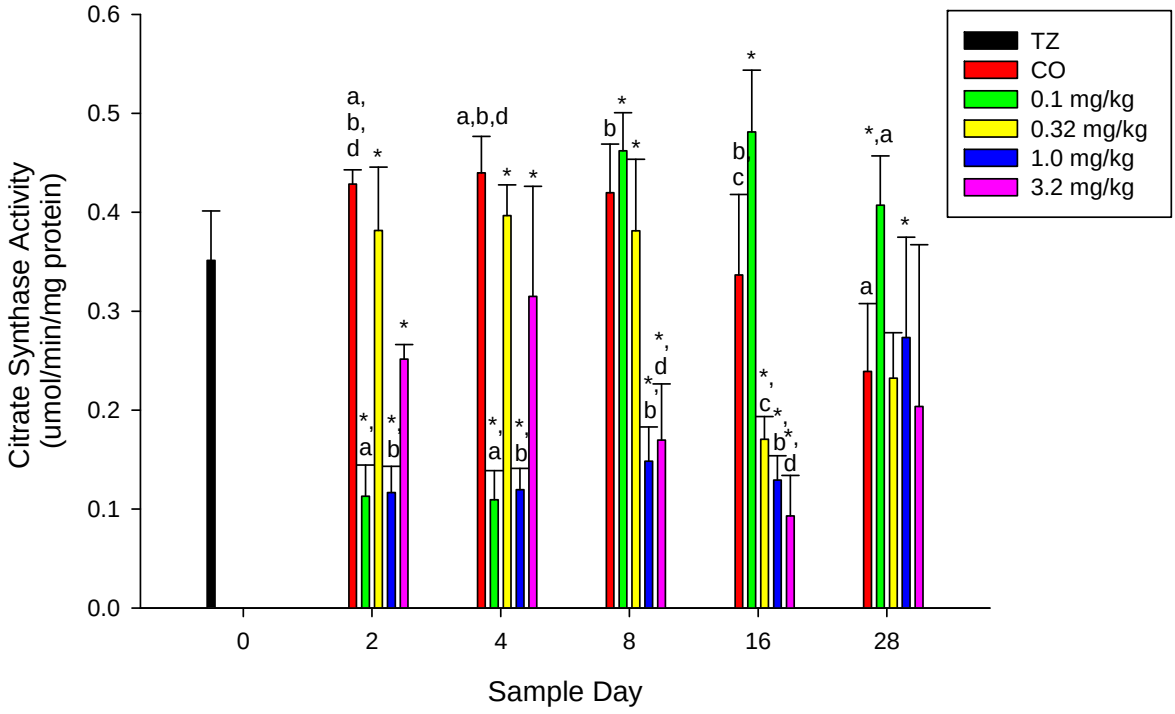

Figure 5.4: Liver CS activity of time zero fish (TZ) and rainbow trout exposed to endosulfan (0.1, 0.32, 1 and 3.2 mg/kg). Data is presented as means ± standard error. Note bars follow the order as appearing in the legend. * denotes significance within treatments across sampling days, a shows the difference between 0.1 mg/kg and corn oil, b is comparison between 1.0 mg/kg and corn oil, c denotes significance between 0.32 mg/kg and d denotes significance between 3.2 mg/kg and corn oil.

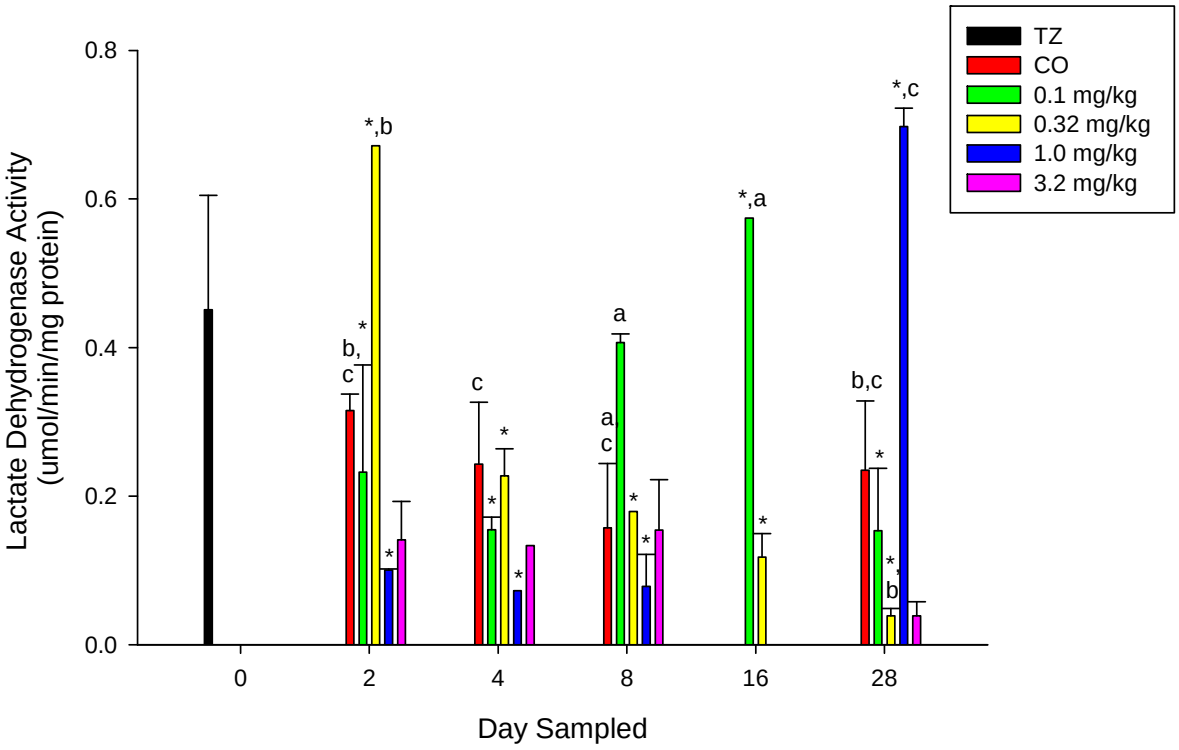

Figure 5.4: Female liver LDH Activity of time zero fish (TZ) and rainbow trout exposed to endosulfan (0.1, 0.32, 1 and 3.2 mg/kg). Data is presented as means ± standard error. Note bars follow the order as appearing in the legend. * denotes significance within treatments across time, letters represent difference in treatments compared to corn oil controls.

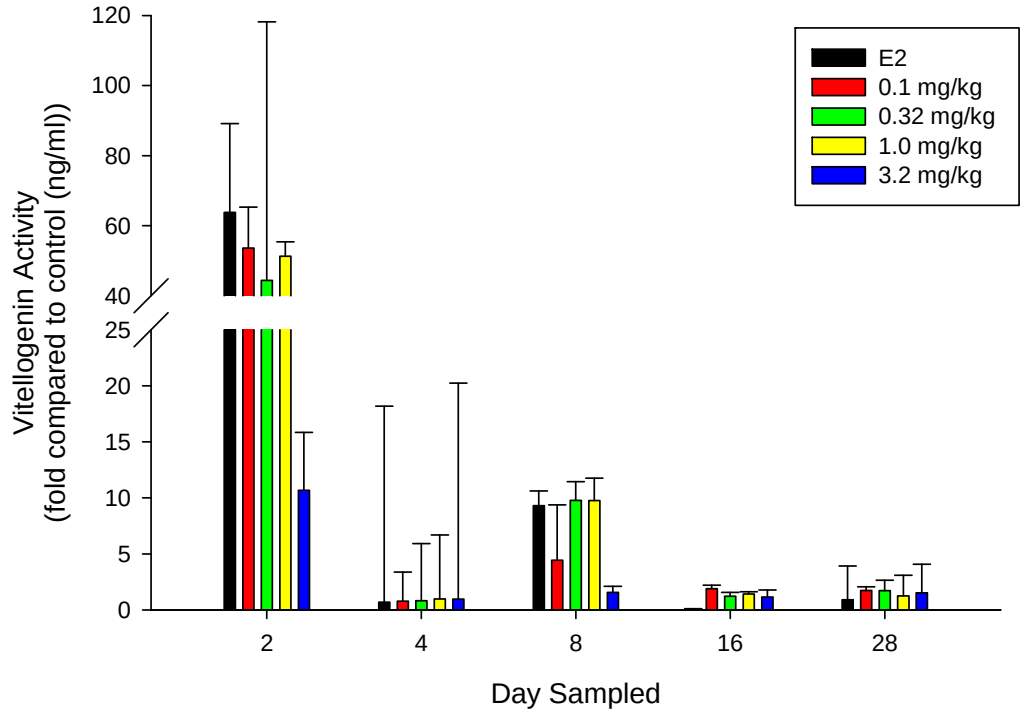

Figure 5.6: Plasma VTG activity of rainbow trout exposed to endosulfan (0.1, 0.32, 1 and 3.2 mg/kg) and 17-β estradiol (E2). Data is given as fold increase over corn oil control. Note bars follow the order as appearing in the legend. Day 2 and 8 are significantly induced compared to corn oil.

5.4.0 Discussion

The inhibited CS levels that were observed for all endosulfan treatments might have affected the fish bioenergetics. A study by Mathers *et al.,* (1993) found that rainbow trout fry fed minimal rations had decreased CS activity (15.56 ± 3.49 µmols/g·DWmin) compared to trout that were fed a medium ration. Thus it could be conceptualized that the decreased CS activity could have been caused by a decrease in ration intake, rather than a direct effect of endosulfan. In future studies fish should be fed to a controlled ration level to remove this possible confounding effect of ration.

Overall liver CS activity was generally lower in trout exposed to endosulfan than to corn oil and 17-β estradiol and a study by Tripathi and Verma (2004) observed that adult freshwater catfish (*C. Batrachus*) exposed to 0.06 mg/L of endosulfan had decreased liver CS activity compared to the controls with a peak decrease of 1.3853 ± 0.582 to a final activity of 2.068 ± 0.344 units/g wt tissue mass at 21 days exposure. Since this was a waterborne application of endosulfan it is not directly comparable to i.p. injections, however it demonstrated that endosulfan had an effect on aerobic metabolism. This study also observed that as the fish were removed from exposure tanks and placed in clean water CS activity along with LDH activity returned to normal, further supporting the hypothesis that endosulfan is quickly eliminated from the body and does not accumulate in the tissues after a pulse waterborne exposure (Tripathi and Verma, 2004; EPA, 2002).

Endosulfan exposure did cause an increase in female liver LDH activity which is in agreement with Kurutaş *et al.,* (2006) who observed increased LDH groupings compared to the controls via histopathological techniques. This increasing trend seen is in agreement to effects seen with rainbow trout fed cyclopropenoid fatty acids and aflatoxin B_1 (Taylor *et al.,* 1973).

Contrary to this, Tripathi and Verma (2004) observed a decrease in brain LDH activity by 31% when freshwater catfish were exposed to 0.06 mg/L endosulfan. The increased female LDH activity seen in this study was however, not larger than the time zero control which could be attributed to the females going through sexual maturation

and requiring more nutrients than they were obtaining from their feed. It has been found that female cardiac LDH activity was 30 % higher than in males (Battiprolu *et al.*, 2006), indicating a possible higher reliance on anaerobic glycolysis. Thus gender must also be considered when investigating metabolic enzymes.

The higher time zero LDH activity could also be attributed to cyclic enzyme induction corresponding to daily feeding times. To possibly reduce this effect, trout should have been starved for a period greater than 24 h to disrupt the cycle (Mathers *et al., 1993*).

Liver EROD activity is used primarily to quantify the induction of cytochrome P-450 1A1 (Petrulis *et al.*, 2000). An unexpected result from this study was the decreased activity of EROD with exposure compared to corn oil controls. This is contrary to several other studies that reported increased activity (Coimbra *et al.*, 2007; Jensen *et al.*, 1991). Fish treated with 3.2 mg/kg endosulfan sampled at day 4 did have a significantly increased EROD activity compared to the other treatments including corn oil. Unfortunately that day correlated to significant death at that time. The decreased EROD could be attributed to the other concentrations being too low to cause a significant induction of EROD or endosulfan being transformed and being eliminated from the body before it could significantly induce EROD activity (Tripathi and Verma, 2004; EPA 2002). Unfortunately EROD is hard to detect at significant levels if there are large variances in baseline levels (Kammann *et al.*, 2005).

There was high variability in the weight and sexual maturation of the fish used in this experiment. For future experiments it would be recommended to individually tag each fish and to decrease fish weight variability by reducing their time in holding tanks before they are to be used and keep their feed maintained (e.g. 4 % body weight) and not fed to satiation. In this particular experiment the trout were held for approximately 4 months before being used and the fish had time to mature. Upon removal there were 4 fish that had mature gonads. To avoid this issue it would be advisable to use the fish within a more appropriate amount of time (2 weeks after collecting from the hatchery). This would reduce the amount of weight and sexual variation seen in this experiment.

Plasma VTG induction from endosulfan exposure was similar to that of 17-β estradiol for all endosulfan doses at sample day 2 (1 – fold increase over corn oil control).

There was no difference between males and females during this time period and increased plasma VTG levels would be indicative of endocrine disruption (Larkin *et al., 2003*). The VTG induction did decline after 2 days for all treatments which is in agreement with 17-β estradiols induction and decline pattern (Orrego *et al.*, 2009).

Increased VTG is characteristic of organochlorine exposure and has been found for several organochlorine compounds (Hodges *et al.*, 2000 and Metcalfe *et al.*, 2000). VTG induction observed in this experiment is contradictory to results found by DHHS (2000) and Harris *et al.* (2000). They observed a decrease in plasma VTG for catfish exposed to 1.5 µg/L endosulfan after 48 hours and no induction of VTG in rainbow trout after 9 days with a single i.p. injection. Harris *et al.*, (2000) proposed endosulfan as an anti-estrogenic compound which would be in agreement with DHHS (2000). If induction of VTG did follow the pattern observed in this experiment then it is quite possible that the observations by DHHS (2000) were too late after injection to see any VTG induction.

It should be noted that there is very little work with VTG measurements for rainbow trout and endosulfan. Thus more work should be done on this aspect to give a more definitive conclusion.

5.5.0 Conclusion

From this experiment it can be concluded that exposure to juvenile rainbow trout via a single i.p. injection with endosulfan can cause increased liver EROD activity, disrupted aerobic and anaerobic metabolism and has the potential to be an endocrine disrupter as evidenced by induced plasma VTG levels. Follow up experiments should be done possibly with use of genomics, to look at VTG expression to confirm its induction by endosulfan acting as an endocrine disruptor as there is conflicting evidence. In future studies it would also be desirable to individually tag fish so changes in weight and length could be determined.

6. Laboratory Endosulfan Exposure 2

6.1.0 Introduction

A second laboratory exposure was conducted which controlled for some of the confounding factors that occurred in the first laboratory exposure. Factors which were addressed included standardizing the trout weights (50-90 g) and adding an additional treatment to account for the possibility of endosulfan acting as an anti-estrogenic compound in rainbow trout (Harris *et al.*, 2000).

The aim of this study was to replicate the original laboratory exposure and to investigate the possibility of endosulfan acting as an anti-estrogenic compound through the use of an endosulfan and 17-β estradiol mixture.

6.2.0 Materials and Methods

6.2.1 Fish

300 rainbow trout (*Oncorhynchus mykiss*) were obtained December 22, 2008 from Rainbow Springs Trout Farm in Thamesford, ON. They were held in 1500 L tanks and maintained at 10.4 ± 0.2 ^{o}C with a dissolved oxygen content of 9.44 ± 0.11 ppm and acclimated in lab for two weeks before use. 164 trout were selected between the weights of 50-90 g for use in this exposure.

6.2.2 Stock and Working Solutions

6.2.2.1 Endosulfan Stock Solution

Endosulfan stock solution was created by weighing 40.3 mg of 2:1, α:β-endosulfan (analytical standard) into a 2 ml micro-centrifuge tube on a micro-balance. In the fume hood 1 ml of acetone was used to dissolve the endosulfan. This was then added to 9 ml of acetone in a 10 ml brown injection bottle. Contents were mixed on a vortex for ~10 s. Final stock solution created was 4.03 mg/ml and stored in a plastic box in the -20 ^{o}C freezer until needed.

6.2.2.2 Endosulfan Working Solutions

From the endosulfan stock solution, 4 working solutions were produced (0.1, 0.32, 1, and 3.2 mg/ml. In each working solution the process was identical except for the amount of corn oil or endosulfan used. Briefly for the 0.1 mg/ml working solution; 0.124 µl of stock solution was combined with 9.88 ml of corn oil in a 10 ml brown injection bottle. The bottle was then capped with a rubber septum and sealed shut by crimping a metal ring around the rubber septum and the top of the bottle. The solution was stored alongside the stock solution in the -20 ^{o}C freezer. For the 0.32, 1, and 3.2 mg/ml working solutions; 0.397, 1.24, and 3.97 ml endosulfan stock solution were combined with 9.60, 8.76, and 6.03 ml of corn oil respectively. All solutions were thawed for 1 hour before use and mixed with a vortex for ~20 s.

6.2.2.3 17-β-estradiol

This solution was prepared as per section 5.2.2.3.

6.2.2.4 Mix Solution

Mix solution was a combination of 0.32 mg/ml endosulfan and 5 mg/ml 17-β estradiol. It was prepared in the same manner as the 0.32 mg/ml with the addition of 50 µg of 17-β estradiol and vortexed until the solution was thoroughly mixed and then stored alongside the other solutions in the -20 ^{o}C freezer.

6.2.3 Injections

Injections commenced on January 14th, 2009 as per section 5.2.2 with the addition of the mixed treatment. The extra treatment added another 20 trout to a total fish count of 164 trout with a weight range between 50-90 g.

Fish were sampled on 0, 2, 4, 8, 16, and 28 days after injection and analyzed as per 4.2.0 – 4.5.0.

6.3.0 Results

Since trout were not tagged individually or separated by tank when sampled final weight and standard length are given for each treatment and sampling period (**Table 6.1** and **Table 6.2**).

As per section 5.3.0 sexes were analysed for differences in the various biomarkers tested. The only biomarker that was significantly affected was liver LDH and only female LDH had significantly different activity. If no difference was detected than data were pooled by sexes and analysis was continued normally. There was no difference in the gonadosomatic index. Condition factor and hepatosomatic index did have significant differences which can be seen in **Figure 6.1** and **Figure 6.2**.

Fish exposed to 0.1 mg/kg endosulfan had a 1.0 – fold increase at day 8 followed by a reduction in condition factor in trout sampled on day 28 of the same magnitude as the original increase. This increase in condition factor was also higher than fish injected with corn oil sampled on days 4-16 by 2.4 ± 0.13 - 1.4 ± 0.05 g/cm^3 respectively. The decrease in condition factor for trout dosed with 0.1 mg/kg endosulfan sampled on day 16 and 28 were below the condition factor for the corn oil control fish at the corresponding days. Condition factor in fish dosed with 0.32 mg/kg endosulfan did not vary significantly over time but was significantly greater than the corn oil controls sampled on day 4 by 0.14 ± 0.1 g/cm^3. Fish exposed to 1.0 mg/kg endosulfan had higher condition

factor than corn oil controls sampled on day 4. Fish dosed with 1.0 mg/kg endosulfan had a 1 – fold lower condition factor at day 8 and 16 compared to fish sampled on day 2 and 28. Trout injected with 3.2 mg/kg endosulfan sampled on day 16 had a higher condition factor than fish sampled on days 4 and 28. Fish dosed with 3.2 mg/kg had a larger condition factor on days 2 through 8 by approximately 1.0 – fold. On day 4, the condition factor of fish exposed to 3.2 mg/kg endosulfan was lower than both 0.32 and 1.0 mg/kg endosulfan treatments by 0.16 ± 0.08 and 0.17 ± 0.07 g/cm^3 respectively.

Mix treatment fish followed the same condition factor pattern as those injected with 3.2 mg/kg endosulfan but, on day 2 their condition factor was significantly higher than fish exposed to 3.2 mg/kg endosulfan on days 2 – 8. Similar to those exposed to 1.0 and 3.2 mg/kg endosulfan, the mix treatment showed a lowering condition factor on sample days 4 – 16 with recovery occurring on days 16 – 28, relative to controls.

Trout exposed to 17-β estradiol and corn oil exhibited the above mentioned profile with those sampled at day 8 having a lowered condition factor compared to fish sampled on days 2 and 28 for 17-β estradiol. Corn oil controls sampled on day 4 had a lowered condition factor compared to fish sampled on day 2.

Trout exposed to 0.1 mg/kg endosulfan increased their hepatosomatic index by 1.0 – fold from sample day 2 to 8. After day 8 the hepatosomatic index of fish dosed with 0.1 mg/kg endosulfan dropped by 1.0 – fold to levels below corn oil controls at days 16 and 28 (**Figure 6.3** and **6.4**). Exposure to 0.32 mg/kg endosulfan followed an increasing trend through all days with those sampled on day 16 (1.0 – fold) having larger HSI than fish sampled on days 2 and day 28. Fish dosed with 0.32 mg/kg sampled on day 28 for the same treatment had a larger hepatosomatic index than all sample days with a 1.5 – fold increase from day 2. HSI of fish injected with 0.32 mg/kg endosulfan was not different from the corn oil controls. Trout injected with 1.0 mg/kg endosulfan had a decreased hepatosomatic index compared to corn oil control fish sampled at day 16. Fish sampled on days 2, 4, 16, and 28 in the third endosulfan treatment (1.0 mg/kg) had a hepatosomatic index lower than the corn oil control fish sampled on day 28. Exposure to 3.2 mg/kg endosulfan displayed an increasing trend in hepatosomatic index starting at day 16 with fish sampled on day 28 being significantly larger than those sampled on days

Table 6.1: Weight of time zero fish (TZ), control rainbow trout (EC) and fish exposed to corn oil (CO), 5 mg/kg 17-β estradiol (E2), endosulfan (0.1, 0.32, 1 and 3.2 mg/kg) and mix solution of 5 mg/kg 17-β estradiol and 0.32 mg/kg endosulfan over 28 days. Values are given as means ± standard deviation.

	TZ	EC	CO	17-B
Time zero	68.14 ± 5.42			
Day 2		55.99 ± 6.72	61.05 ± 10.63	58.30 ± 13.70
Day 4		59.73 ± 15.43	61.14 ± 8.79	60.73 ± 7.78
Day 8		67.46 ± 8.76	65.49 ± 10.50	68.78 ± 13.84
Day 16		59.89 ± 16.39	63.84 ± 3.63	72.05 ± 10.30
Day 28		92.69 ± 15.67	76.19 ± 14.67	73.70 ± 8.48

	0.1	0.32	1	3.2	MIX
Day 2	61.50 ± 11.31	71.05 ± 7.28	67.62 ± 11.89	53.16 ± 3.23	66.86 ± 14.80
Day 4	56.93 ± 10.18	72.18 ± 14.11	71.16 ± 9.87	77.55 ± 5.38	55.75 ± 6.46
Day 8	54.34 ± 1.74	75.05 ± 9.33	70.24 ± 10.67	62.79 ± 14.22	68.65 ± 7.37
Day 16	68.49 ± 10.71	67.45 ± 4.97	66.46 ± 6.69	74.60 ± 11.82	58.43 ± 6.73
Day 28	60.86 ± 10.62	74.74 ± 12.28	70.33 ± 4.18	80.57 ± 14.95	70.20 ± 4.18

Table 6.2: Standard length of time zero fish (TZ), control rainbow trout (EC) and fish exposed to corn oil (CO), 5 mg/kg 17-β estradiol (E2), endosulfan (0.1, 0.32, 1 and 3.2 mg/kg) and mix solution of 5 mg/kg 17-β estradiol and 0.32 mg.kg endosulfan over 28 days. Values are given as means ± standard deviation.

	TZ	EC	CO	17-B
Time zero	16.75 ± 0.289			
Day 2		16.15 ± 1.06	16.25 ± 0.55	15.80 ± 1.53
Day 4		16.40 ± 1.36	17.00 ± 0.77	16.15 ± 0.44
Day 8		16.55 ± 0.53	16.95 ± 1.34	17.28 ± 1.53
Day 16		15.93 ± 1.48	16.78 ± 0.26	17.25 ± 0.96
Day 28		18.38 ± 0.85	17.13 ± 0.63	17.13 ± 0.85

	0.1	0.32	1	3.2	MIX
Day 2	16.50 ± 1.29	17.28 ± 0.63	16.75 ±1.19	15.88 ± 0.48	16.50 ± 0.46
Day 4	16.18 ± 0.54	17.35 ± 1.64	17.20 ± 1.13	18.50 ± 0.41	16.08 ± 0.25
Day 8	15.40 ± 0.20	17.65 ± 0.72	17.48 ± 0.92	16.83 ± 1.39	17.58 ± 0.53
Day 16	17.33 ± 0.70	16.78 ± 0.91	17.38 ± 0.48	17.63 ± 1.25	16.25 ± 0.32
Day 28	17.00 ± 0.82	17.25 ± 0.87	17.00 ± 0.71	17.63 ± 1.03	17.13 ± 0.24

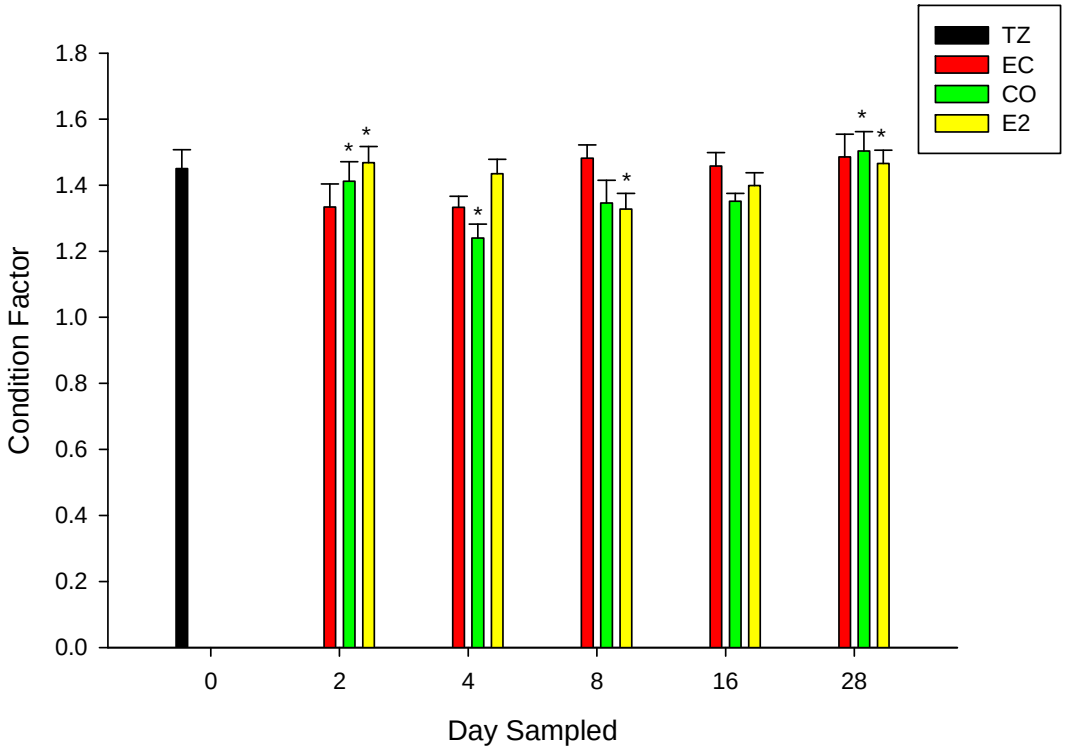

Figure 6.1: Condition factor of time zero fish (TZ) and control trout (EC) along with fish injected with corn oil (CO) and 17-β estradiol (E2). Values are given as means ± standard error. Note bars follow the order as appearing in the legend. * represents significance within treatments across time.

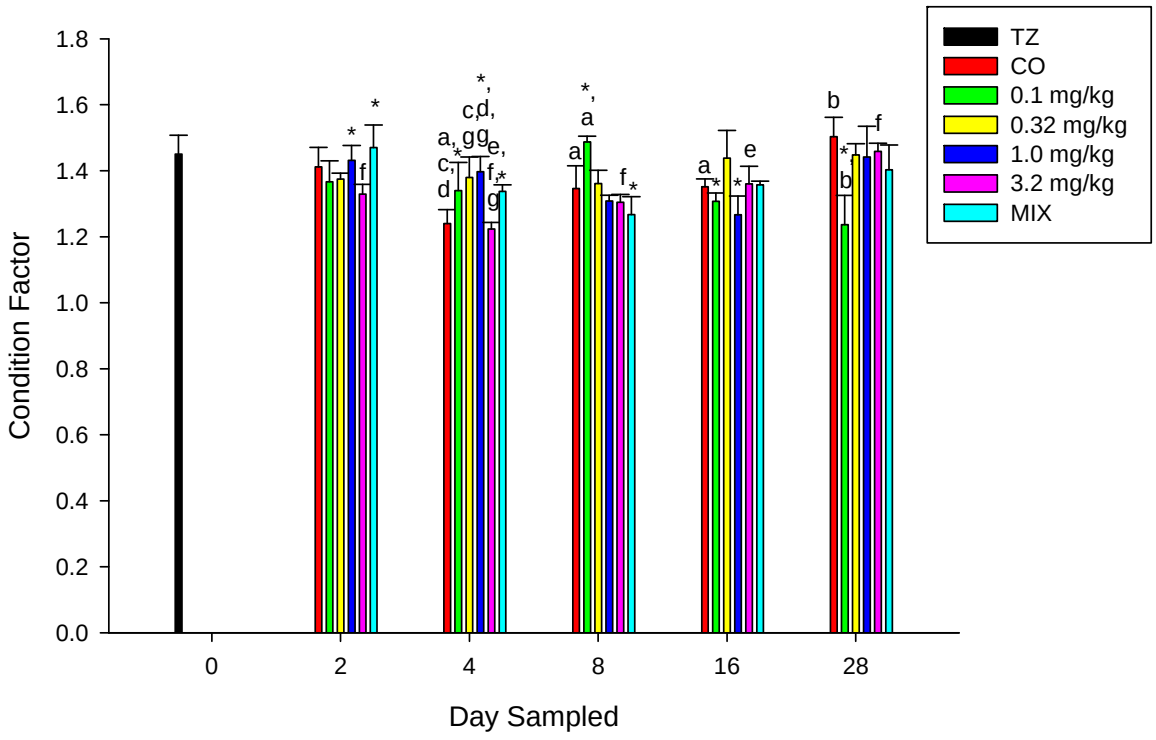

Figure 6.2: Condition factor of time zero fish (TZ) and rainbow trout exposed to endosulfan (0.1, 0.32, 1 and 3.2 mg/kg) and a mix of 5mg/kg 17-β estradiol and 0.32 mg.kg endosulfan. Values are given as means ± standard error. Note bars follow the order as appearing in the legend. * represents significance within treatments across time, a, c, d denotes significance between 0.1, 0.32, 1.0 mg/kg and corn oil respectively, b compares 0.1 mg/kg and corn oil on day 28, e and f compares days 4 and 16 and days 4, 8, and 28 for 3.2 mg/kg respectively, lastly g denotes significance between 0.32 mg/kg and 1.0 mg/kg with 3.2 mg/kg.

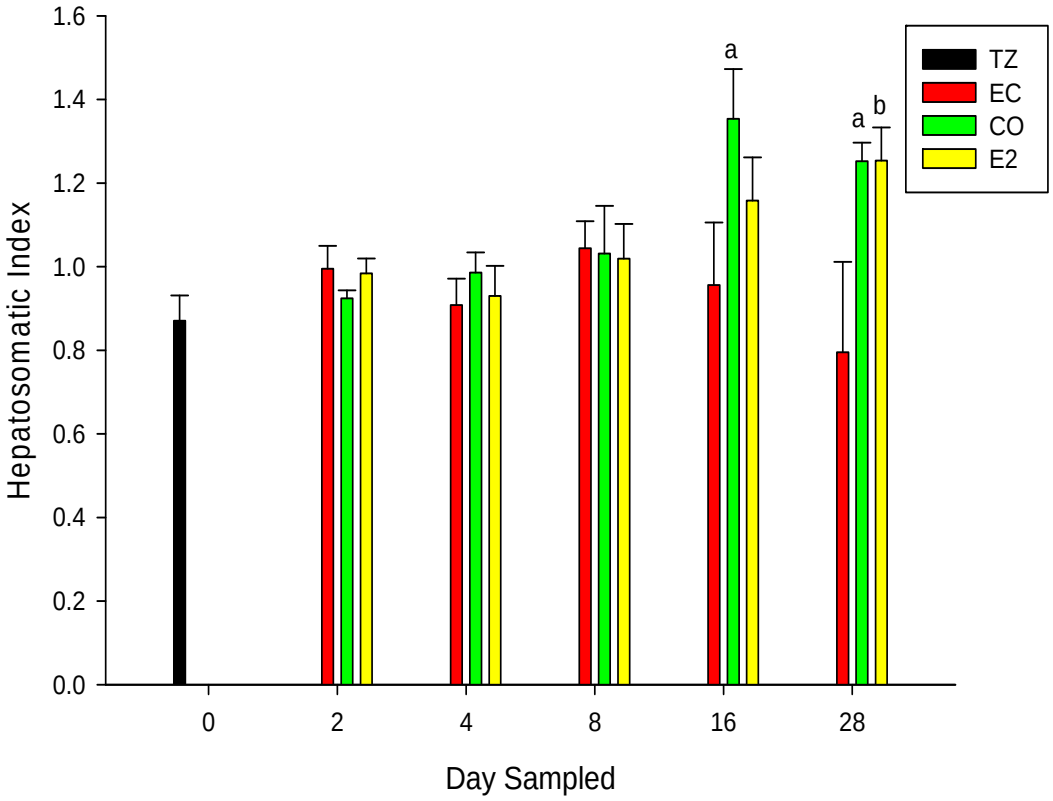

Figure 6.3: Hepatosomatic of time zero fish (TZ) and control trout (EC) along with fish injected with corn oil (CO) and 17-β estradiol (E2). Values are given as means ± standard error. Note bars follow the order as appearing in the legend. a and b represents significant difference of corn oil compared to experimental control across all sample days.

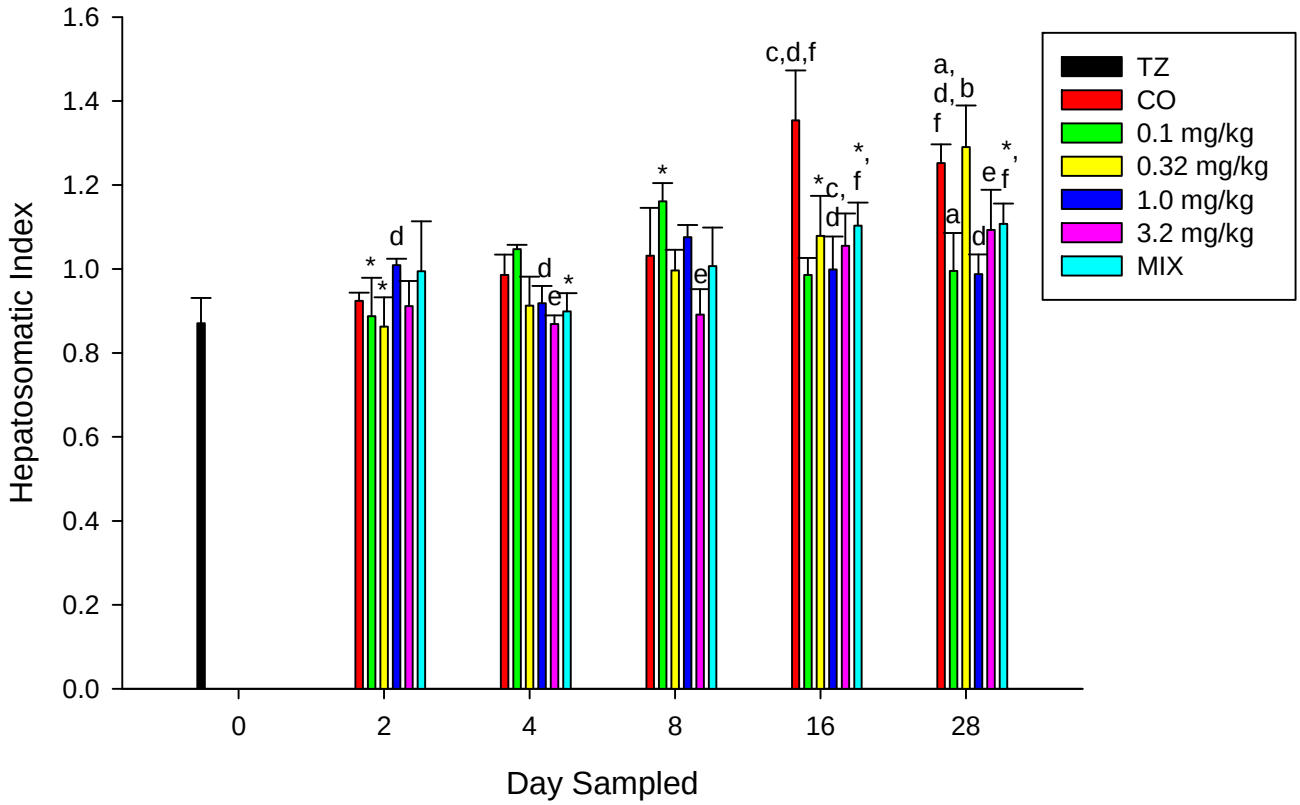

Figure 6.4: Hepatosomatic index of time zero fish (TZ) and rainbow trout exposed to endosulfan (0.1, 0.32, 1 and 3.2 mg/kg) and a mix of 5 mg/kg 17-β estradiol and 0.32 mg/kg endosulfan. Values are given as means ± standard error. Note bars follow the order as appearing in the legend. * represents significance within treatments across time, a indicates difference of 0.1 mg/kg with corn oil at day 28, b represents significance of 0.32 mg/kg across all sample days, c and d show difference with 1.0 mg/kg compared to corn oil on day 16, e denotes significance of 3.2 mg/kg, days 2 and 8 with day 28, and f signifies significance of the mix treatment with corn oil on the corresponding days.

4 and 8. Corn oil control fish HSI was also larger at day 16 than HSI of fish treated with 3.2 mg/kg endosulfan sampled on days 2-16.

Fish exposed to the mixed treatment also displayed an increasing trend in hepatosomatic index at day 8 and like the other treatments HSI decreased at day 16 and 28 compared to corn oil controls. Mix treatment fish had a total increase in hepatosomatic index of 0.11 ± 0.17 g/cm^3 over the 28 days.

Corn oil control fish had a significant increase in hepatosomatic index at day 16 and 28 compared to fish sampled on days 2 and 4. This was a 1.5 – fold increase for those 2 days. Fish injected with corn oil had a maximum increase in hepatosomatic index of 0.56 ± 0.33 g/cm^3 over the experimental controls.

Liver citrate synthase activity was generally reduced by endosulfan with CS of 0.1 mg/kg injected fish not different over time, but liver CS activity of all endosulfan injected fish sampled on days 8 - 28 had 2.0 to 4.5 – fold lowered activities compared to the respective corn oil controls (**Figure 6.5** and **Figure 6.6**). CS activity of fish injected with 0.32 mg/kg endosulfan did not vary significantly over the 28 day sampling period. CS of controls increased by 0.21 ± 0.09 μmol/min/mg protein over the experimental period reaching 0.44 ± 0.09 μmol/min/mg protein at the 28 day sampling period.

Fish injected with the 17-β estradiol – 0.32 mg/kg endosulfan mixed treatment followed the same CS activity pattern as all the endosulfan treatments where CS activity was not different over time but was significantly lower for fish sampled on days 2 – 28 compared to the controls sampled on days 8 – 28.

Female liver lactate dehydrogenase activity in trout was also affected by exposure to endosulfan (**Figure 6.7** and **Figure 6.8**). Females injected with 0.1 mg/kg endosulfan had a 26 and 23 – fold induction of LDH activity on days 2 and 28 compared to 0.1 mg/kg injected fish sampled on day 16, respectively. LDH activity of 0.1 mg/kg injected fish not higher than controls on the corresponding days. Fish injected with 0.32 mg/kg endosulfan had no difference in LDH activity across all sample days, but were

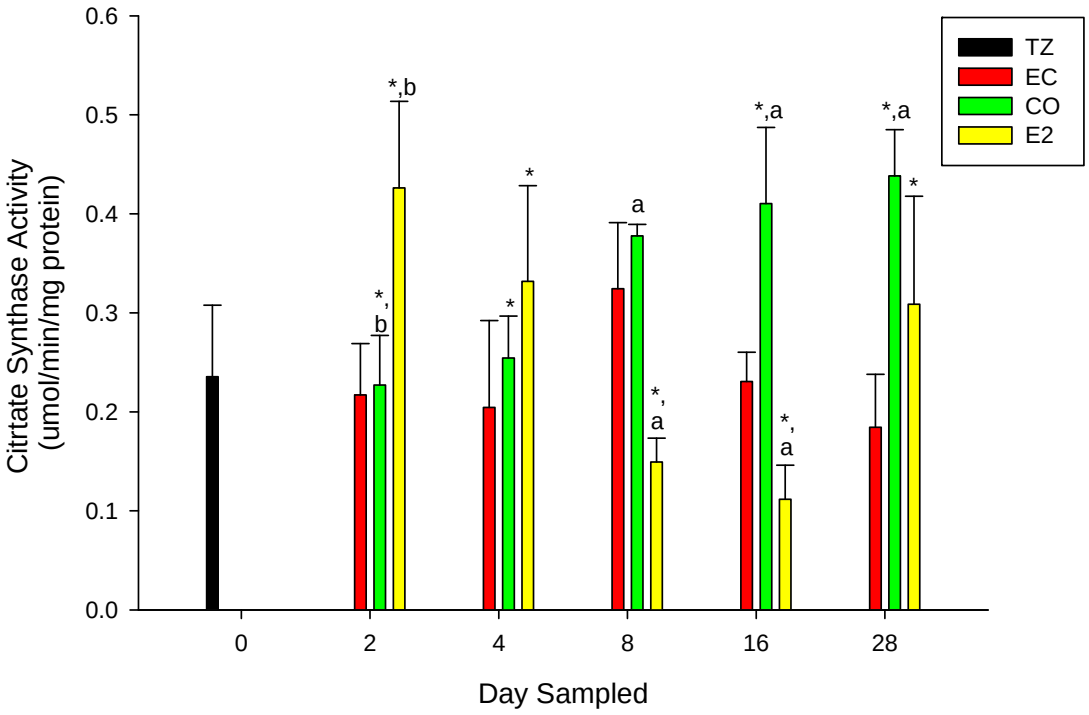

Figure 6.5: Liver CS activity of time zero fish (TZ) and control trout (EC) along with fish injected with corn oil (CO) and 17-β estradiol (E2). Values are given as means ± standard error. Note bars follow the order as appearing in the legend. * denotes significance within treatments across time, a represents difference of 17-β estradiol compared to corn oil, and b represents the same thing as "a" but only for day 2.

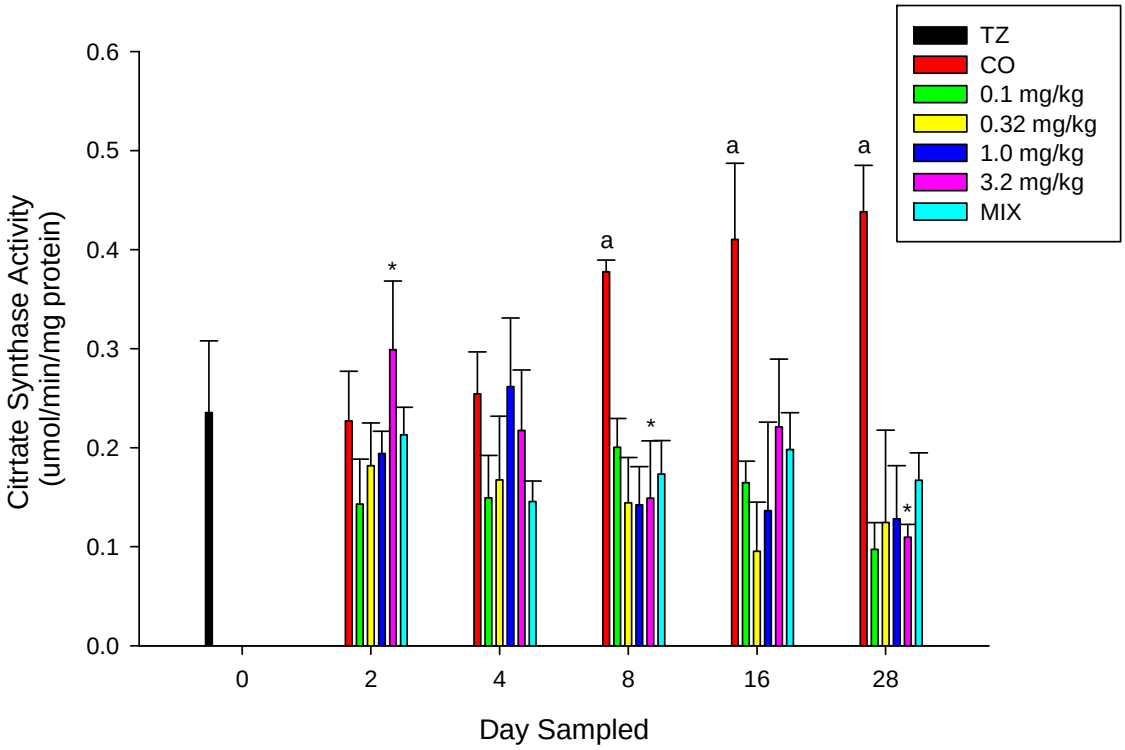

Figure 6.6: Liver CS activity of time zero fish (TZ) and trout exposed to endosulfan (0.1, 0.32, 1 and 3.2 mg/kg) and a mix of 5 mg/kg 17-β estradiol and 0.32 mg/kg endosulfan. Values are given as means ± standard error. Note bars follow the order as appearing in the legend. * represents significance within treatments across time, a denotes significance across all treatments and time except for 1.0 mg/kg, day 4 compared to corn oil on day 8 and 3.2 mg/kg at day 2 with corn oil on days 8 and 16.

significantly lower than day 16 corn oil controls which had LDH activity of 1.33 μmol/min/mg protein. Trout dosed with 1.0 mg/kg endosulfan had a significantly larger liver LDH activity on day 8 than at all other sample periods, but their LDH activity was not different from corn oil controls on any sample day. Fish injected with 1.0 mg/kg endosulfan had the highest LDH activity (1.00 ± 0.39 μmol/min/mg protein) compared to the other endosulfan and mix treatments. Exposure to 3.2 mg/kg endosulfan did not produce any significant induction of LDH across all sample days. Fish sampled on days 8 and 16 from the 3.2 mg/kg endosulfan treatment had significantly inhibited LDH activity compared to corn oil controls sampled on days 2, 16, and 28. Trout dosed with 3.2 mg/kg endosulfan sampled on day 8 had lower LDH activity than fish treated with 0.1 and 0.32 mg/kg endosulfan sampled on day 1. Trout injected with 3.2 mg/kg endosulfan also had lower LDH activity compared to fish treated with 0.1 mg/kg endosulfan sampled on day 28.

A significant decrease in LDH activity was observed in mixed treatment fish sampled on days 8 (2.5 – fold) and day 28 (320 – fold) compared to corn oil controls. Mix treated fish sampled at day 4 had a significant induction of LDH activity compared to fish sampled on day 28.

Time zero fish had a larger liver LDH activity compared to all treatments and sampling times except for fish dosed with 1.0 mg/kg endosulfan sampled on day 8.

Within controls (**Figure 6.7**), corn oil treated fish sampled at day 16 had the largest induction of LDH activity compared to all the other controls except for time zero fish. The next largest activity was in livers of experimental controls sampled on day 4 at 0.73 ± 0.16 μmol/min/mg protein.

Trout injected with endosulfan were observed to have plasma VTG induction relative to corn oil controls compared to all other treatments across all sample days (**Figure 6.9**). Corn oil controls also had the highest induction of VTG (966.1 ± 37.1 ng/ml). Fish treated with the mix treatment had increased VTG induction compared to all treatments except corn oil controls, day 28 experimental controls, and those treated with 0.32 mg/kg endosulfan. Trout treated with 17-β estradiol had increased VTG induction

for days sampled on days 2, 8, and 16 compared to fish treated with 0.32 mg/kg endosulfan by 2 – fold.

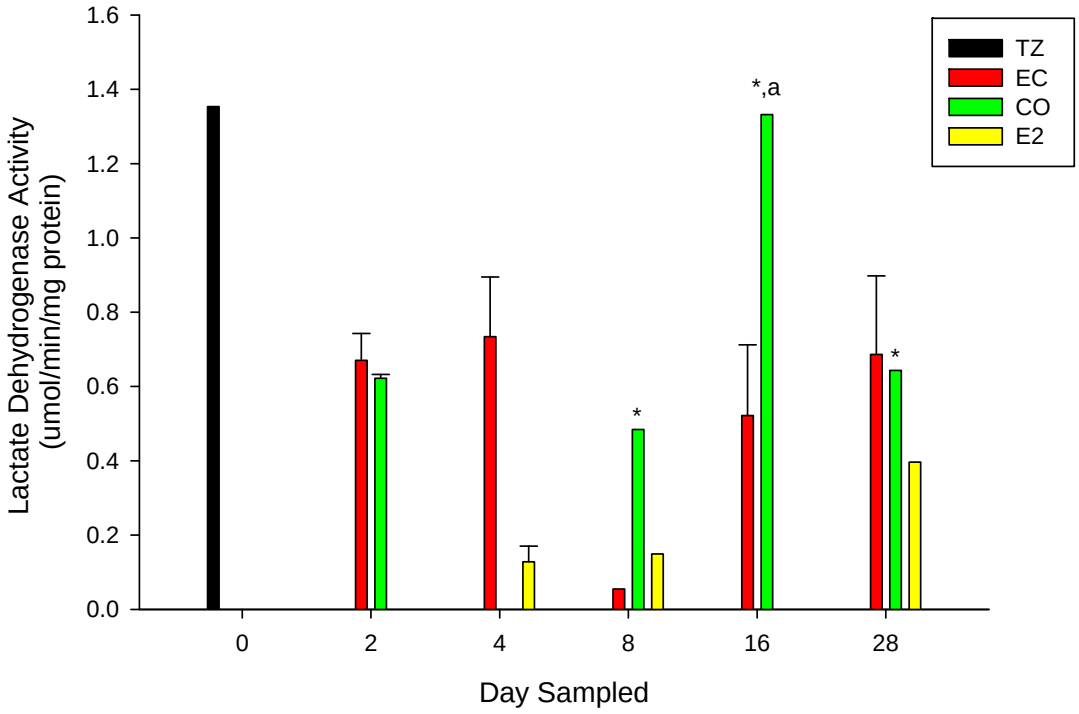

Figure 6.7: Female liver LDH activity of of time zero fish (TZ) and control trout (EC) along with fish injected with corn oil (CO) and 17-β estradiol (E2). Values are given as means ± standard error. Note bars follow the order as appearing in the legend. * denotes significance within treatment across time and a represents significance across all days and treatments, except with time zero.

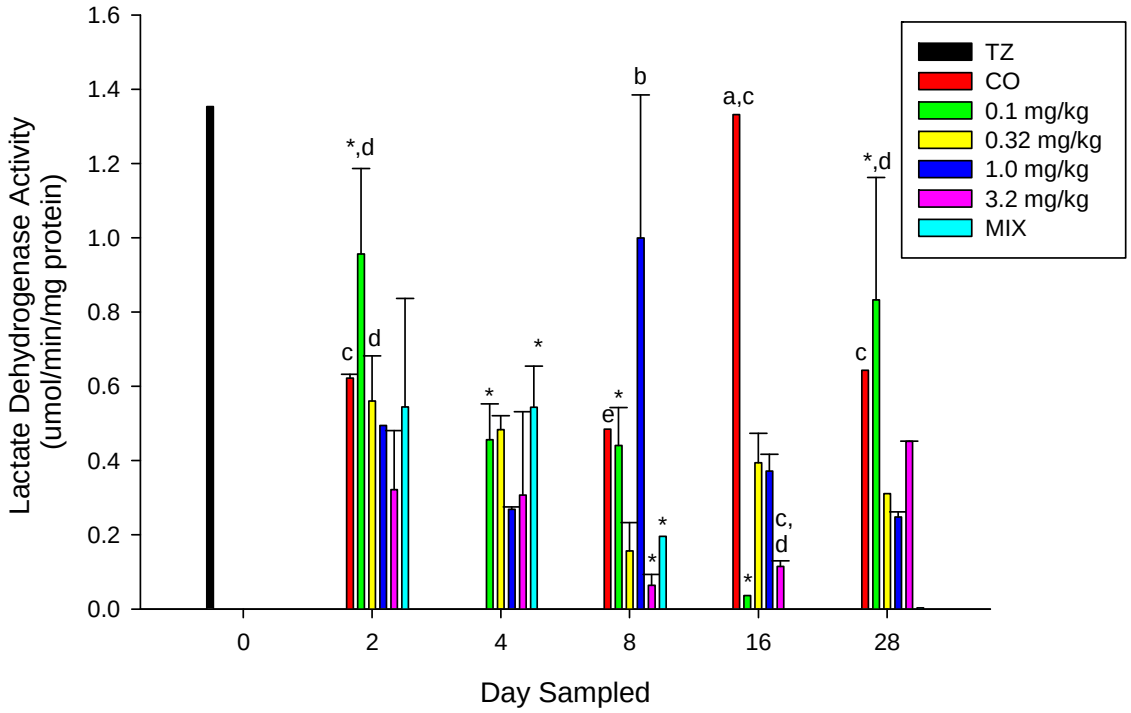

Figure 6.8: Female liver LDH activity of time zero fish (TZ) and trout exposed to endosulfan (0.1, 0.32, 1 and 3.2 mg/kg) and a mix of 5 mg/kg 17-β estradiol and 0.32 mg/kg endosulfan. Values are given as means ± standard error. Note bars follow the order as appearing in the legend. * denotes significance within treatments across time, a indicates significance across time and treatments except compared to 1.0 mg/kg at day 8, b represents significance across all time periods for 1.0 mg/kg, c shows significant difference of 3.2 mg/kg on day 16 with corn oil on days 2, 16 and 28, d indicates a difference between 3.2 mg/kg at day 16 with 0.1 mg/kg and 0.32 mg/kg on day 2 and 0.1 mg/kg at day 28.

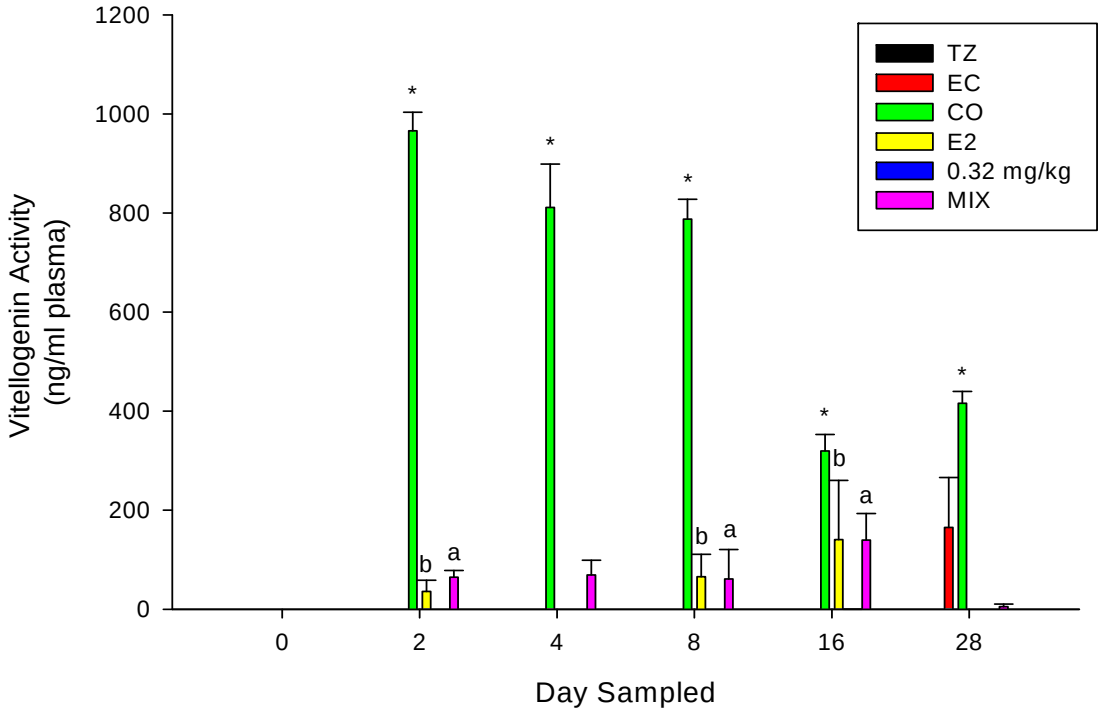

Figure 6.9: Plasma vitellogenin activity of time zero fish (TZ) control rainbow trout (EC) and trout exposed to corn oil (CO), 0.32 mg/kg endosulfan and a mix of 5mg/kg 17-β estradiol and 0.32 mg.kg endosulfan. Values are given as means ± standard error. Note bars follow the order as appearing in the legend. * denotes significance of fish treated with corn oil compared to all other treatments at all sampling days. a represents significance on fish treated with mix treatment compared to experimental control fish those treated with 0.32 mg/kg endosulfan sampled at days 2, 8 and 16. b denotes significance between fish treated with 17-β estradiol and fish treated with 0.32 mg/kg endosulfan on days 2, 8, and 16.

6.4.0 Discussion

Citrate synthase activity was significantly inhibited in all treatments in fish livers sampled on days 8-28. This observation is an indication that endosulfan caused a decrease in aerobic metabolism which was also seen in the first laboratory endosulfan exposure (Section 5.3.0). However, there was not an increase in female liver LDH activity across all treatments or in males. There was only increased LDH in trout sampled on days 2 and 8 for 0.1 and 1.0 mg/kg endosulfan treated trout respectively. This was contrary to what would be thought to occur when aerobic metabolism is inhibited. LDH activity results are different to what was found in the first endosulfan experiment. Females in both experiments had a significant difference in liver LDH compared to males, similar to results observed by Battiprolu *et al.,* (2006) but, in the first experiment there was a noticeable increase in liver LDH activity in contrast to this experiment.

In the first experiment the trout were significantly larger than those used in this exposure. A study by Somero and Childress (1980) observed an increase in muscle LDH activity from 5 different sampling points for *Paralobrax clathratus* and 9 sampling spots along the dorsal side of *Medialuna californiensis* with increasing fish size. Even though this is in dorsal muscle tissue it could be indicative that the smaller trout used in the second experiment did not have the potential to induce anaerobic metabolic rates compared to the larger fish used in the first laboratory exposure.

Rats fed a diet of 1.0 and 2.0 mg/kg of endosulfan per day experienced a 1.3 and 1.6 fold increase in testicular LDH respectively (Sinha *et al.,* 2001). Although involving testes, it is apparent that endosulfan caused an increase in LDH activity in the test organisms in various tissues. Thus it is possible that injected trout were able to metabolize endosulfan and remove it before anaerobic metabolism was necessary.

Exposure to endosulfan caused a decrease in condition factor followed by recovery, except for the lower doses which experienced a slow decline. This decline and recovery could be indicative of endosulfan being removed from the body (EPA, 2002). A decrease in condition factor was also observed in white sturgeon (*Acipenser transmontanus*) exposed to various organochlorine chemicals in the USA (Foster *et al.,*

2001). The decrease in condition factor could be caused by endosulfan reducing fat stores of the trout exposed. Decreased fish weight was reported following exposure to varying simultaneous exposures to endosulfan and disulfoton (Arnold *et al.,* 1995). At the highest exposure concentrations (50 ng/L endosulfan and 10 µg/L disulfoton), trout experienced a 50 % decrease in weight compared to controls over the 34 day exposure. A decrease in fish weight can lead to a decrease in condition factor as observed in this experiment.

There was no correlation of endosulfan dose and increased HSI or liver EROD which was also observed in some experiments with Nile tilapia (*Oreochromis niloticus*) exposed to 0.001 - 1.0 µg/g endosulfan (Coimbra *et al.,* 2007). Trout exposed to endosulfan experienced increases followed by decreases in HSI. The increase in HSI is most likely related to the liver increasing CYP 1A1 protein production to detoxify the injected endosulfan followed by possible liver damage as endosulfan persisted or increased endosulfan concentrations were administered. It is this increasing of production of CYP 1A1 followed by possible necrosis that might cause the liver to increase in size without inducing significant levels of EROD activity (Coimbra *et al.,* 2007). This type of result has also been seen in rainbow trout with other pesticides (Paraquat) (Fifueiredo-Fernandes *et al.,* 2006).

The increased liver EROD activity and lack of HSI significance in the first endosulfan experiment could have resulted from all endosulfan concentrations not causing irreparable liver necrosis, thus allowing the trout to continue to produce CYP 1A1 without any inhibitory effects (Coimbra *et al.,* 2007). This could in large part be due to the larger fish that were used in the first experiment since they are more tolerant to endosulfan exposure (Capkin *et al.,* 2006).

Fish treated with 0.32 mg/kg endosulfan and mix treatment showed little difference in induction of plasma VTG activity. It has been thought that endosulfan acts an endocrine disruptor, possibly acting as an anti-estrogenic compound. It is possible that at the concentration tested in the mix treatment was not strong enough to cause an anti-estrogenic effect. The large induction of VTG in fish treated with corn oil is contradictory to what other studies observed including; Orrego *et al.,* (2009) and Aït-Aïssa *et al.,* (2003) who observed decreased VTG levels compared to 17-β estradiol treatment. The

VTG results for corn oil controls were also contradictory to those found in the first laboratory experiment. Fish injected with corn oil had a 7 – fold induction relative to the previous experimental corn oil controls. This variation could be due to contaminated corn oil or an error in the running of the VTG assay. Future studies could use a different immersing agent instead of corn oil and VTG expression could be determined by gene expression instead of an ELISA.

Compared to the first laboratory experiment, this experiment has produced conflicting results, specifically the difference in liver LDH activity and high control plasma VTG. To counter act this effect it would be recommended to repeat the experiment again at concentrations of interest with standardized fish weights. In this case it would be advisable to repeat with trout that were 50-90 g as there was less variability in their final weights. Another modification would also be increasing the starvation period before sampling to ensure enzyme induction with feeding is disrupted and not able to skew the results. Finally, it would be essential to tag individual fish to permit growth changes to be determined.

6.5.0 Conclusion

From this experiment it can be concluded that endosulfan disturbs aerobic and anaerobic metabolism as seen with the inhibited liver CS levels and slight increase in liver LDH activity. Endosulfan also caused an increase in liver EROD activity but not an increase in HSI. There was however a decrease and recovery in condition factor over the 28 day sample period. Lastly endosulfan treated fish compared to the mix and 17-β estradiol treatment did not confirm that endosulfan causes an anti-estrogenic effect in rainbow trout with no differences in plasma VTG levels. Further studies should be implemented that look at more diverse mix treatments to determine the effects of endosulfan as an endocrine disrupting chemical.

7. Oshawa Creek Field Exposure 1 (August –September 2008)

7.1.0 Introduction

Oshawa Creek Watershed is located in Southern Ontario, Canada. It is a spring fed river system composed 8 sub-watersheds that eventually empty out into Lake Ontario at Oshawa Harbour (CLOCA 2002). Oshawa Creek is primarily composed of gravel, sand, silt, clay, and muck types of sediment which could potentially bind and retain harmful chemicals (Hose *et al.,* 2003; EPA, 2002). This could hold primary importance due to the variable land use that is contained within the Oshawa Creek Watershed. To the North the land is used for primarily agricultural purposes with scattered horse farms. Moving South the land is used for residential and then finally commercial and industrial platforms closer to Lake Ontario (CLOCA 2002; CLOFMP 2007). With the varied use surrounding Oshawa Creek it is quite viable that there are at least one point source of contaminants entering the Creek, be it from agricultural (pesticides), residential (sewage and garbage), or industrial processes (heavy metals) providing a variety of toxicologically relevant effects.

Besides the varied land use within the watershed, Oshawa Creek is home to a variety of aquatic life including; american brook lamprey (*Lampetra appendix*), blacknose dace (*Rhinichthys atratulus*), brook (*Salvelinus fontinalis*), brown (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), chinook Salmon (*Oncorhynchus tshawytscha*), and pike (*Esox luciu*) (CLOFMP 2007). In particular the salmonids use Oshawa Creek as a breeding ground both in the fall (salmon) and in the spring (trout). If there were toxicants in the water it is possible that they could affect the most susceptible life-stages and in turn decrease their populations.

It is the aim of this study to preliminarily characterize the West and Main Branch of Oshawa Creek with caged rainbow trout (*Oncorhynchus mykiss*) during the early fall

of 2008. An off stream reference site was used which was located in Black Creek. Blood plasma VTG, liver EROD, LDH, CS, and brain AChE were assessed to determine potential evidance of toxicity.

7.2.0 Materials and methods

7.2.1 Fish

Rainbow trout (*Oncorhynchus mykiss*) were obtained on May 7th, 2008 from Rainbow Springs Trout Farm in Thamesford, ON. The batch contained 247 trout (197 ± 64 g). They were held in 1500 L tanks and maintained at 11.8 ± 0.23 ^{o}C with a dissolved oxygen content of 9.5 ± 0.19 ppm and acclimated in lab for two weeks before use.

7.2.2 Cages

7.2.2.1 Original Cage Design

Trout cages were constructed out of 61x30x41 cm Rubbermaid© containers with 1.9 cm holes were bored out of all four sides. 0.635 cm drill bit was used to drill 4 holes into the bottom of the container, along the upper rim of the container and 4 adjoining holes in the lid. Total of 10 holes were drilled around the perimeter of the lid (3 along each side and two on the handles) to secure the lid to the bottom. The lid and bottom were connected by zip ties. A viewing window (17 cm x 7 cm) was cut into the center of the lid and secured closed with a zip tie. Another 4 holes were drilled with the 0.635 cm bit in a rectangle pattern (21 cm x 10.5 cm) in which an identification sign was attached with zip ties. The sign was made out of bright yellow plastic and a laminate covering to protect the print. The modified Rubbermaid© containers were then attached to two cinder blocks via 60 cm zip ties crisscrossed through the bottom of the containers in the aforementioned holes (**Figure 7.1**).

7.2.2.2 Cage Trial 1

Two cages were placed in the field at each site on July 8th and recovered on July 22nd-August 8th. The cages were removed from the field due to excessive rains and subsequent cage damage and losses (77.7 % damaged, 22.2 % missing, and 11.1 % destroyed).

7.2.2.3 Cage Modification 1

The previous steps were used in this modification except that the 4 holes drilled into the bottom of the cages were replaced with 4, 1.27 cm holes and an additional 12, 0.635 cm holes were drilled into the bottom around the perimeter. Two plastic plates (31 cm x 23 cm) with matching holes to the bottom of the cage were used to reinforce the bottom (one inside, one outside). They were attached to the bottom of the cage with black zip ties through the 12, 0.635 holes. The cages were then attached to the two cinder blocks via 60 cm zip ties lengthwise through the 1.27 cm holes in the bottom of the cage (**Figure 7.2**). The two cinder blocks were then connected to each other with 2, 60 cm zip ties (one at each end). A 121.92 cm length of chain was then fed around the center support of the cinder blocks and connected with a quick link. Attached to the chain was a 10.16 cm metal ring. Once in the water the metal ring was attached to a length of rope (varied depending on site) which connected both cages and then secured to a shore based structure (tree) approximately 2 m away from the river bank.

7.2.2.4 Cage Trial 2

Cages were replaced in the field on August 11th, 2008 at above mentioned sites.

7.2.3 Cage Locations

Locations for placing cages were determined mainly on where UOIT had pre-existing permits for experimentation in the Oshawa Creek Watershed. Once this was determined the second factor considered was if the river was deep enough to have the cages completely submerged with about 30 cm of extra headspace. This was accomplished by visiting the different sites and wading into the water with chest waiters and getting an approximate depth. A third criterion was to attempt to have all the sites on Oshawa Creek kept as far apart as possible (minimum 10 km) to prevent overlap of exposures. A fourth criterion was the ease at which the site was accessible by foot. From this criterion, three sites were decided upon in Oshawa Creek (**Figure 7.3**) and one in Black Creek (offsite reference). Oshawa Creek sites were located (North to South) near Simcoe St. and Columbus Rd. (43.979 N, 78.925 W), Simcoe St. and Conlin Rd. (43.946 N, 78.904 W), and Simcoe St. and Thomas St. (43.869 N, 78.846 W). The offsite reference site was located near Taunton Rd. and Hancock Rd. (43.953 N, 78.786 W).

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Figure 7.1: Original cage design depicting an interior and exterior view of the cages used in the fall exposure.

Figure 7.2: Cage modification 2 depicting reinforced bottom with plastic platting and increased number of holes around the exterior.

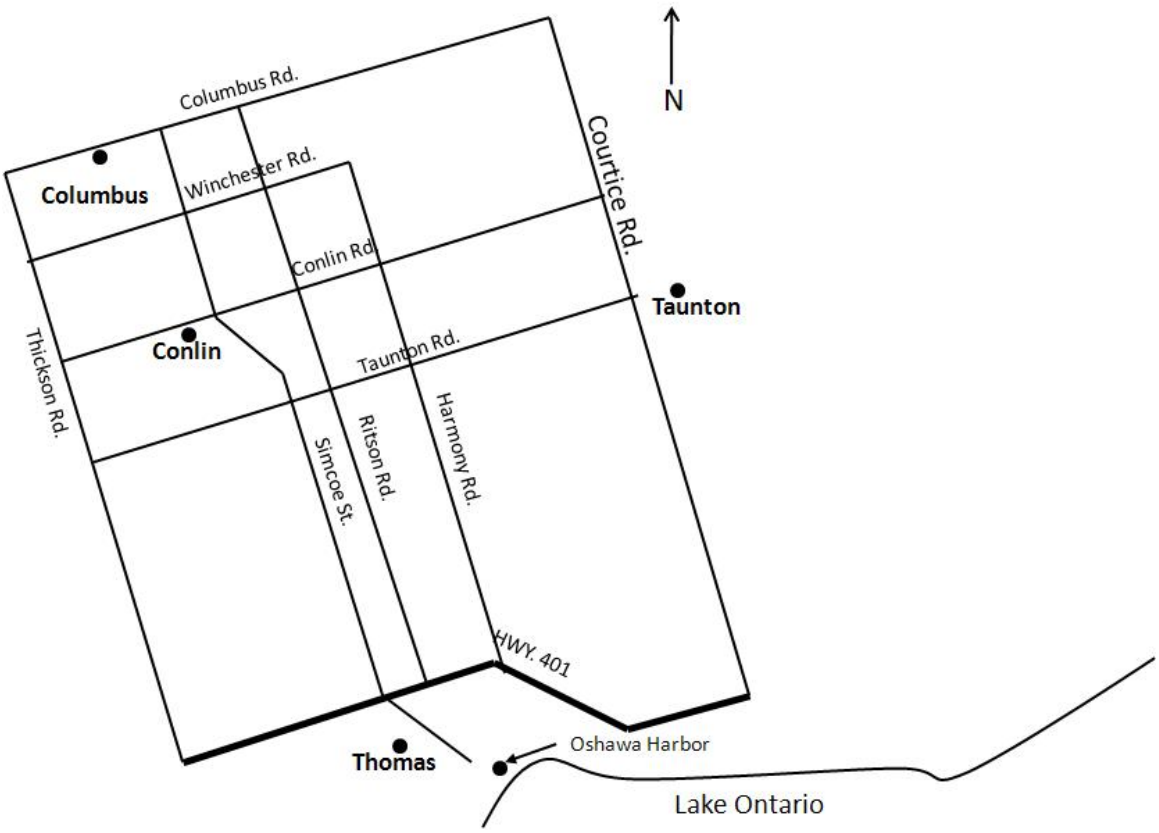

Figure 7.3: Locations of cages in the Oshawa Creek Watershed during the fall exposure.

7.2.4 Field Exposure

Trout field exposure was initiated on August 19, 2008. The trout were transported to the various field locations in plastic garbage buckets (with lids) with battery powered air systems connected to two air stones in the bucket. The buckets were secured in the truck with bungee cords (one around the bucket and one holding the lid on). Once in the field, the buckets were moved near the cages, the bungee cord and lid removed. Trout were then acclimated to field water with river water. After acclimation, the trout were lifted from the bucket with a 25 cm x 20 cm net and put into the cages through the window on the top of the cage. The window was then re-secured with a black zip tie. The trout remained in the field until September 18th when they were brought back to the lab in a similar fashion as to when they were taken out. They were then anaesthetized and sampled as per 4.2.0.

7.2.5 Abiotic Field Measurements

7.2.5.1 Flow Rate

Flow rates were measured at each site on days 0, 14, and 28. This was accomplished by placing a cork ring in the water and recording how long it took to travel one meter. Flow rate was measured and reported in seconds per meter in triplicate.

7.2.5.2 Dissolved Oxygen

Dissolved oxygen (DO) was measured with a La Motte dissolved oxygen kit (Winkler modification) on days 0, 14, 28. Briefly, a 50 ml water sample was collected in 50 ml glass bottle ensuring there were no air bubbles. Then 8 drops of manganous sulphate were added followed by 8 drops of alkaline potassium iodide azide solution. The bottle was capped and gently inverted 8-12 times. This produced a yellow/brown flocculent that was allowed to settle below the neck of the bottle before 8 drops of 1:1 sulfuric acid were added. The bottle was then inverted again until the flocculent

disappears (the sample was now fixed). The sample was then stored on ice until analyzed back in the lab (up to 8 h of storage). Once back in the lab 20 ml of the sample was transferred to a 50 ml holding tube (provided), a 10 ml syringe (provided) was filled with sodium thiosulphate (0.025 N) and was used to titrate the fixed sample until a pale yellow colour appeared. Eight drops of starch indicator solution was added turning the sample blue and titration continued until the blue colour disappeared. The amount of sodium thiosulphate used was directly proportional to the amount of dissolved oxygen in the water in parts per million.

7.2.5.3 Alkalinity, Hardness, pH, Nitrate and Nitrite

All measurements were taken at once using a Quick Dip test strip manufactured by: Jungle: fish care made easy. The strip was dipped into the water and held level for 30 s, and then alkalinity, hardness, pH, and nitrite were read. After 60 s nitrate was read. These concentrations were determined by comparing the test strip the legend provided with the test strips.

7.2.5.4 Total Organic Carbon

Sediment samples were gathered at each caging site on days 0, 14, and 28. They were collected with a 50 ml Falcon tube and stored on ice until transported back to the lab. Once back in the lab they sat at room temperature for 2 days until the head water was removed and disposed of. Samples were then stored in the -80 ^{o}C freezer until further analyzed.

7.2.5.5 Thermogravimetric Analysis

TGA analysis was carried out on TA Instruments SDT Q 600 Thermal Analyzer with Q series software for data analysis. Sediment was allowed to thaw for 24 h and then placed in the aluminum crucible at a weight of 68.954 ± 14.805 mg wet weight. The

samples were then heated to determine the percent weight that was composed of organic materials. This was performed with the following design; sampling interval of 1.0 s/pt, run sample under dry air at 50 ml/min, data storage turned off, equilibrate at 120 ^{o}C, isothermal for 10 minutes, turn on data storage, heat ramp 20 ^{o}C/min to 600 ^{o}C, turn flow rate to 0 ml/min, turn on air cool.

TOC was also determined by the simpler method of TGA. Samples (10 g) were dried for ~24 h at 120 ^{o}C to remove any water content. The samples were then transferred into ceramic crucibles (5 g of sample) and placed in a Fisher instruments muffle furnace (550 ^{o}C) until there was no weight change (measured at ~3 h intervals). Change of initial mass (entering furnace) and end of furnace treatment was used as a measure of total organic carbon.

7.2.6 Tissue Analysis

7.2.6.1 Brain Acetylcholine Esterase (AChE)

Brain AChE analysis was performed using a modified version of the procedure described by Sandahl and Jenkins (2002). Brains were sectioned so that 4 mg of brain was immersed in 156 µl of 100 mM sodium phosphate buffer with 1 % triton X-100 (1 in 39 volumes). They were then homogenized with an Ika: T-25 basic Ultra-Turrax homogenizer. Homogenates were then centrifuged at 1000 g for 10 minutes at 4 ^{o}C, supernatants removed and stored at -80 ^{o}C.

The 100 mM sodium phosphate buffer (pH 8.0) was composed of two other solutions (dibasic and monobasic stock solutions). Dibasic stock solution was composed of 26.807 g of sodium dibasic phosphate (Na_2HPO_4-heptahydrate) dissolved in 1 L of Milli-Q water and stored at 4 ^{o}C. Likewise the monobasic stock solution was composed of 13.8 g of 0.1 M NaH_2PO_4 dissolved in 1 L Milli-Q water and stored at 4 ^{o}C. Sodium phosphate buffer was then prepared by mixing 385 ml of dibasic stock and 15 ml of monobasic stock solution. pH was then adjusted to 8.0 by adding dibasic stock if pH was below 8.0 and monobasic stock if pH was above 8.0.

An aliquot of 99 ml of sodium phosphate buffer was then combined with 1 ml of Triton X-100 to produce the buffer the brains were homogenized in.

Brain supernatants were thawed and 50 µl were transferred to 1.5 ml micro-centrifuge tubes containing 900 µl of 100 mM sodium phosphate buffer. A mass of 7.926 mg of DTNB was dissolved in 2 ml of 100 mM sodium phosphate buffer and 50 µl was added to each micro-centrifuge tube. The tubes were then incubated at room temperature for 10 minutes to allow non-enzymatic activity to stabilize. Once the incubation period was completed 200 µl were transferred in triplicate to a 96 well UV microplate starting at well B1 (Wells A1-3 were used for blanks containing 250 µl of sodium phosphate buffer). An additional 50 µl of Sodium phosphate buffer was added to samples in columns 1, 4, 7, and 10. Reaction was initiated by the addition of 50 µl of acetylcholine iodide (AtChI) to all sample wells except for samples in columns in 1, 4, 7, and 10 (AtChI was prepared by dissolving 13.014 mg of AtChI in 4.5 ml of sodium phosphate buffer). Plate was immediately read for absorbance at 412 nm at 12 s intervals for 10 minutes at 25 ^{o}C.

7.2.6.2 Other Tissue Analysis

All other tissue analysis were conducted in accordance with sections 4.2.0 – 4.5.0

7.3.0 Results

Abiotic factors listed above can be found in **Table 7.1** along with caged rainbow trout characteristics (**Table 7.2**). The only significantly different characteristics found were water temperature and water flow; there was also no significant difference in total organic content in the soil between sites. Taunton had significantly lower water flow (almost 3 – fold slower than Columbus) and temperature compared to the other three sites.

It should be noted that over the 30 day exposure 4 fish died and 2 escaped; 3 died from Thomas and one from Taunton. The 2 fish that escaped, escaped during the initial caging (Conlin and Thomas) and were not replaced. There were numerous fish observed with sores on their ventral sides.

SPMDs were not analyzed for this experiment based on that SPMDs near the Oshawa land fill revealed no toxicologically relevant data.

As with the laboratory exposures the different biomarkers were analysed to see if differences occurred due to sexes and if so were analysed separately. If not then the data was pooled and the analysis continued. There were three differences due to sexes which included EROD, weight and hepatosomatic index. Interestingly, these along with AChE were the only biomarkers that were significantly affected.

Female weight and HSI were not significantly affected while male weight and HSI were and are described in **Table 7.3**. Since fish were not weighed or tagged individually initially final weights are given per site.

Female liver EROD was also significantly affected (**Figure 7.4**) with time zero liver EROD being 2-3 fold higher than trout liver EROD at all the cage sites. The fish caged at Taunton had increased liver EROD activity compared to the trout at Columbus and Thomas sites.

There was no difference in plasma VTG activity in caged trout, thus data will not be shown.

Brain acetylcholine esterase was also significantly affected in both female and male trout (no difference due to sexes) (**Figure 7.5**). Trout at Taunton and Thomas had decreased AChE levels compared to time zero by approximately 2 – fold and to trout at the Conlin site (1.5 – fold). Fish caged at Taunton also had decreased levels compared to trout at Columbus.

Table 7.1: Descriptive abiotic factors for field sites on Oshawa Creek and off stream reference site (Black Creek). Values are given as means ± standard deviation.

	Water flow (s/m)	Water Temperature (°C)	Dissolved oxygen (ppm)	Alkalinity (ppm)
Columbus	3.44 ± 1.50	15.40 ± 1.94	9.65 ± 0.47	300 ± 0
Conlin	6.58 ± 1.17	16.05 ± 1.64	10.10 ± 0.28	300 ± 0
Thomas	8.03 ± 3.68	17.07 ± 1.83	9.74 ± 0.83	300 ± 0
Taunton	14.6 ± 12.7[a]	13.62 ± 1.27[b]	9.80 ± 0.42	300 ± 0

	Hardness (ppm)	Nitrite (ppm)	Nitrate (ppm)	pH	Total Organic Content (g)
Columbus	268 ± 63	1.9 ± 1.3	8.1 ± 3.8	8.4 ± 0	5.00 ± 0.03
Conlin	300 ± 0	2.5 ± 0	10 ± 0	8.3 ± 0.2	5.06 ± 0.06
Thomas	300 ± 0	4.4 ± 3.8	7.6 ± 4.8	8.3 ± 0.3	5.07 ± 0.10
Taunton	262 ± 75	1.9 ± 1.3	10 ± 0	8.3 ± 0.4	5.52 ± 0.27

*superscripts in common denote no significant difference.

Table 7.2: Descriptive rainbow trout characteristics for caged fish. Values are given as means ± standard deviation.

	n	Weight (g)	Standard Length (cm)	Condition Factor (g/cm^3)
Time zero	5	233.7 ± 64.3	25.8 ± 1.8	1.3 ± 0.2
Columbus	10	152.5 ± 54.2	23.4 ± 2.5	1.1 ± 0.2
Conlin	9	191.5 ± 56.6	25.6 ± 2.1	1.1 ± 0.2
Thomas	7	179.7 ± 45.7	24 ± 2.4	1.3 ± 0.1
Taunton	9	162.4 ± 37.7	23.7 ± 1.91	1.2 ± 0.1

	Hepato somatic Index (%)	Gonado somatic Index (%)
Time zero	0.828 ± 0.078	0.067 ± 0.017
Columbus	1.11 ± 0.127	0.118 ± 0.023
Conlin	0.13 ± 0.090	0.094 ± 0.020
Thomas	0.937 ± 0.122	0.226 ± 0.158
Taunton	1.214 ± 0.210	0.088 ± 0.019

Table 7.3: Male weight and HSI for caged rainbow trout in Oshawa Creek and off stream reference site (Black Creek). Values are given as means ± standard deviation.

	Weight (g)	**HSI (%)**
Time Zero	259.2 ± 33.1^{ab}	8.4 ± 0.7^{de}
Columbus	105.3 ± 23.8^{c}	4.2 ± 1.0
Conlin	198.3 ± 29.0^{b}	6.0 ± 1.1^{e}
Thomas	175.8 ± 27.0^{b}	8.4 ± 1.6^{de}
Taunton	163.4 ± 22.8^{bc}	5.6 ± 0.6

*superscripts in common denote no significant difference.

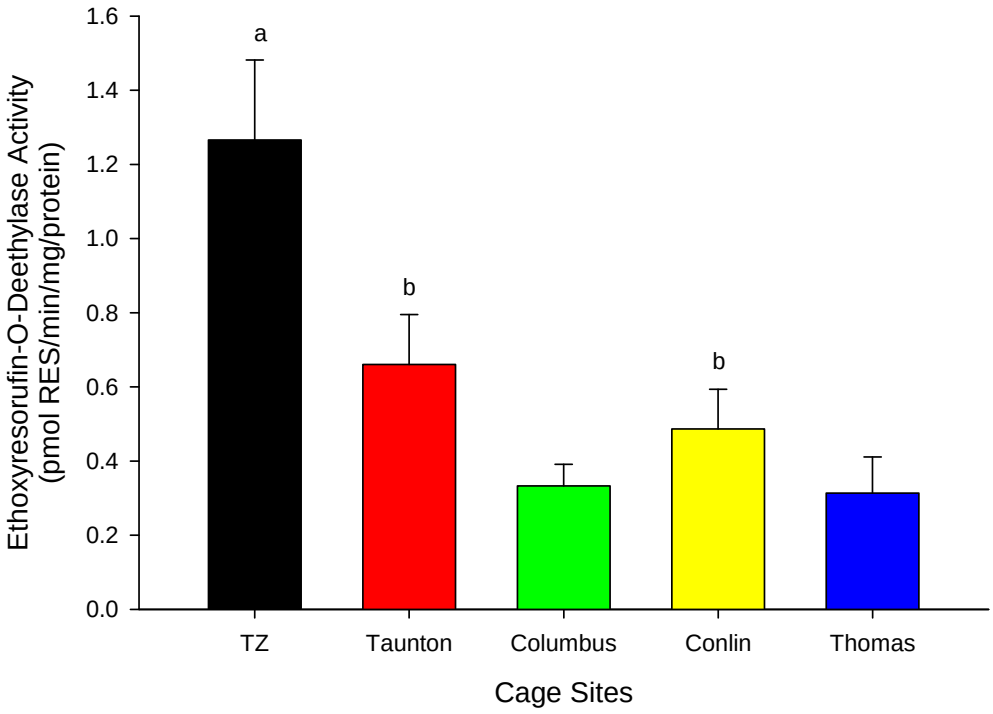

Figure 7.4: Female liver EROD activity of time zero fish (TZ) and to trout caged in Oshawa Creek and the off stream reference site, Black Creek. Values are given as means ± standard error. a denotes significance between time zero and trout at all other cage sites. b represents increased EROD activity for trout at Taunton and Conlin compared to fish at Columbus and Thomas. Letters in common signify no significant difference.

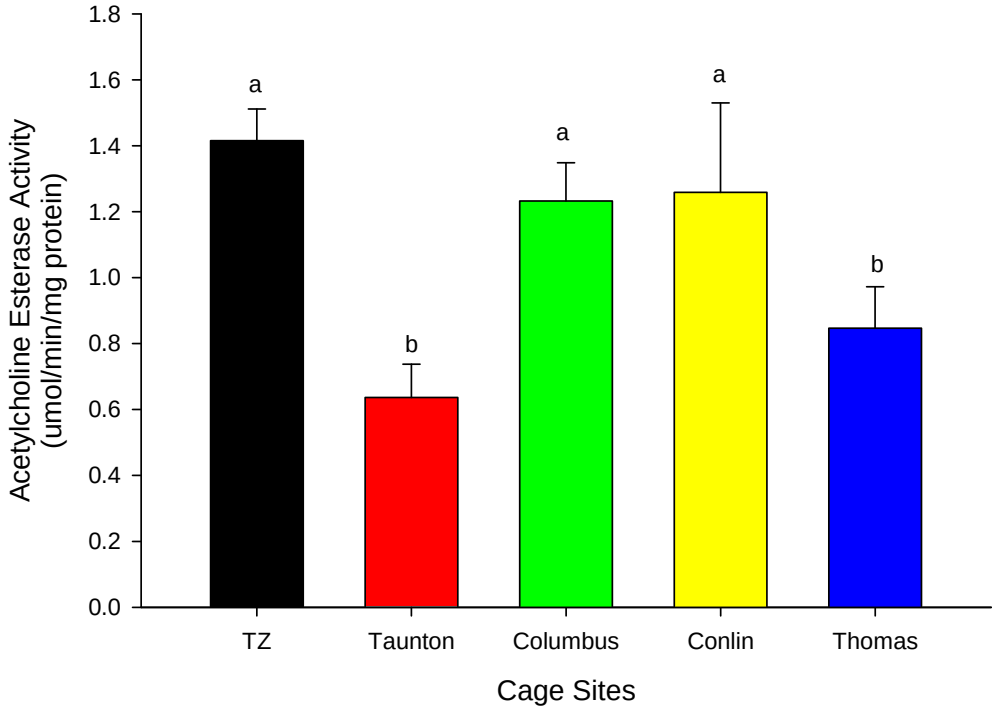

Figure 7.5: Brain AChE activity of time zero fish (TZ) and trout caged in Oshawa Creek and the off stream reference site, Black Creek. Values are given as means ± standard error. a indicates significance between time zero fish and those at Columbus and Conlin with fish caged at Taunton and Thomas. b denotes fish caged at Taunton had significantly decreased AChE activity compare to Columbus and Conlin but not to Thomas. Letters in common signify no significant difference.

7.4.0 Discussion

Trout at Taunton and Conlin sites had increased weight compared to the Columbus and Thomas sites. Their increased weight could be due to those sites receiving higher nutrient sources then the other sites. Taunton site is located at the base of a culvert creating a large circulating pool that can have an influx of nutrients after rain events. Conlin on the other hand is the most Northerly site with rocky patches just upstream of the cages. After rain events or wildlife movement across the stream, macro-invertebrates or insect larvae could be dislodged and travel downstream to the cages.

There is another possibility that could account for the variability in male weight. The trout used in this experiment were originally obtained from the hatchery in May 2008 and housed in the lab until their use in August 2008 due to poor weather conditions. During this time, the trout had ample time to increase weight and become more variable from each other. This type of size differentiation can be described by the observations made my McLaughlin (2001). In that study, two types of feeding behaviour in brook charr were observed (active foraging and sit and wait). The active foragers in turn were able to acquire more nutrients then the sit and wait fish. This type of behaviour of fish housed in confined conditions where food is only administered to the top of the tank can result in decreased food reaching the trout at the bottom of the tank and increased fish weight variability.

It is odd that there was a significant HSI increase in fish at Thomas but there was no significance in male EROD. Thomas could have an increased HSI due to increased mixture of chemicals that the trout were exposed to (agricultural, residential, commercial and industrial sources). In this case it is possible that the trout were being exposed to chemicals that did not cause liver necropsy and allowed the liver to produce enough CYP 1A1 at low enough concentrations to manage the toxicity (Coimbra *et al.,* 2007; Kammann *et al.,* 2005). It should also be noted that the trout being exposed were on average 200 g and already had a well developed liver.

It is also possible that at the other sites, trout were being exposed to chemicals that could cause sub-lethal toxicity or liver necrosis and inhibiting CYP 1A1 production

and EROD activity (Coimbra *et al.,* 2007; Ma *et al.,* 2005). If this were the case then the trout at Columbus, Conlin, and Taunton would not have the ability to detoxify the chemicals and decreased health and higher mortality would have been expected along with increased serum sorbitol dehydrogenase which was not measured. Fortunately this was not the case.

Fish caged at Conlin and Taunton sites had increased female liver EROD activity compared to the other sites but, was lower than time zero trout. Trout at Taunton could have experienced increased EROD activity due to pesticide exposure from the surrounding farm lands. This type of induction has been seen with endosulfan and other pesticides (EPA, 1999; Harris *et al.,* 2000; Brumley *et al.,* 1995). Fish at Conlin experienced an increase in EROD activity. The surrounding area is predominantly agricultural and horse farms, thus trout at Conlin could have experienced the same type of exposure as seen in those caged at Taunton. Trout at Conlin also had a higher stream flow than Taunton, allowing for less exposure time after a potential pulse exposure and thus possibly lower EROD activity.

AChE activity was inhibited in brain of trout at Taunton and Thomas. These effects are seen when acetylcholine esterase is deactivated and acetylcholine cannot be broken down into choline and acetyl-Coa (Pope *et al.,* 2005; Malomouzh and Nikol'skii, 2007). These effects have been observed with carbamate pesticides among other chemicals including; organochlorines, chlorpyrifos, and tetraethyl pyrophosphate and in various aquatic species including; Asian swamp eel, pacific steelhead, goldfish, and rainbow trout (Sandhal and Jenkins, 2002; Liu *et al.,* 2007; Pope *et al.,* 2005; Siang *et al.,* 2007). Effects seen in fish at the Taunton site might thus be attributed to its location and surrounding agricultural land as previously described. Thomas had the second slowest water flow out of the four sites and was the furthest downstream. Since this site is furthest downstream, it is exposed to agricultural, residential and industrial discharges, all of which could have caused the observed decrease in brain AChE activity. Decreased AChE activity can cause convulsions, paralysis and death. Paralysis can lead to increased predation by aquatic, terrestrial, and avian sources and could lead to decreased populations.

As was previously mentioned, there was high variability due to weight. To get more accurate results it would be advised for any future work to reduce variability in weight by using similarly sized experimental fish. To achieve this, it is recommended that holding time be reduced before use and that fish be fed a constant amount of feed (4 % body weight per day) instead of being fed to satiation. If the fish were to be housed for an extended period then the fish should be individually tagged so variation in weight and size a can be tracked on a per fish basis.

The sores observed on the caged trout were most likely caused by their bellies rubbing against the zip ties used to hold the cage to the cinder blocks or to hold the plastic plates to the bottom of the cages. The trout would have also been forced against the zip ties during times of increased current or if the cages were lifted too far out of the water while checking for mortality.

7.5.0 Conclusion

Fish caged in Oshawa Creek along with the off stream reference site (Black Creek) experienced increased female liver EROD activity along with decreased brain AChE activity. Since most of the surrounding area is dedicated to agriculture it is possible that the observed effects were due to agricultural runoff. The variability in weights likely was caused by the increased holding time in the lab before use and should be avoided for future experiments. Lastly, more cage modifications will be needed to ensure they are not interfering with the health of the trout (sores observed on ventral side of fish).

8. Oshawa Creek Field Exposure 2 (May 2009)

8.1.0 Introduction

The first field exposure was pseudo-replicated during the spring of 2009. The same sites were used except for the off stream reference site due to higher public traffic and disturbance through the area. This replication will allow for seasonal effects to be compared across the various sites and within different branches of Oshawa Creek.

The aim of this study was to primarily characterize the West and Main branch of Oshawa Creek with caged rainbow trout (*Oncorhynchus mykiss*) during early spring of 2009. An off stream reference site was used which was located in the East branch of Oshawa Creek. Blood VTG, liver EROD, liver LDH, liver CS, and brain AChE were investigated to determine potential causes of toxicity.

8.2.0 Materials and Methods

8.2.1 Fish

300 rainbow trout (*Oncorhynchus mykiss*) were obtained December 22, 2008 from Rainbow Springs Trout Farm in Thamesford, ON. They were held in 1500 L tanks and maintained at 10.4 ± 0.2 ^{o}C with a dissolved oxygen content of 9.44 ± 0.11 ppm and were held in the lab for 4 months before use in this experiment. A total of 53 fish were used in this experiment.

8.2.2 Cages

The same cages from the first field experiment were used with slight modifications. In this modification the cages were kept the same from the first modification except for the addition of more 1.9 cm holes drilled along all four sides of the container to about 1-2 cm from the bottom. This helped improve water flow through the cage and reduce sediment build-up inside the cage (**Figure 7.2**).

8.2.3 Cage Locations

The same locations were used as per section 7.2.3 except for the off stream reference site. The off stream reference site was moved to inside Camp Samac (43.565N, 78.531W) (**Figure 8.1**) due to increased traffic and fishing at the original site.

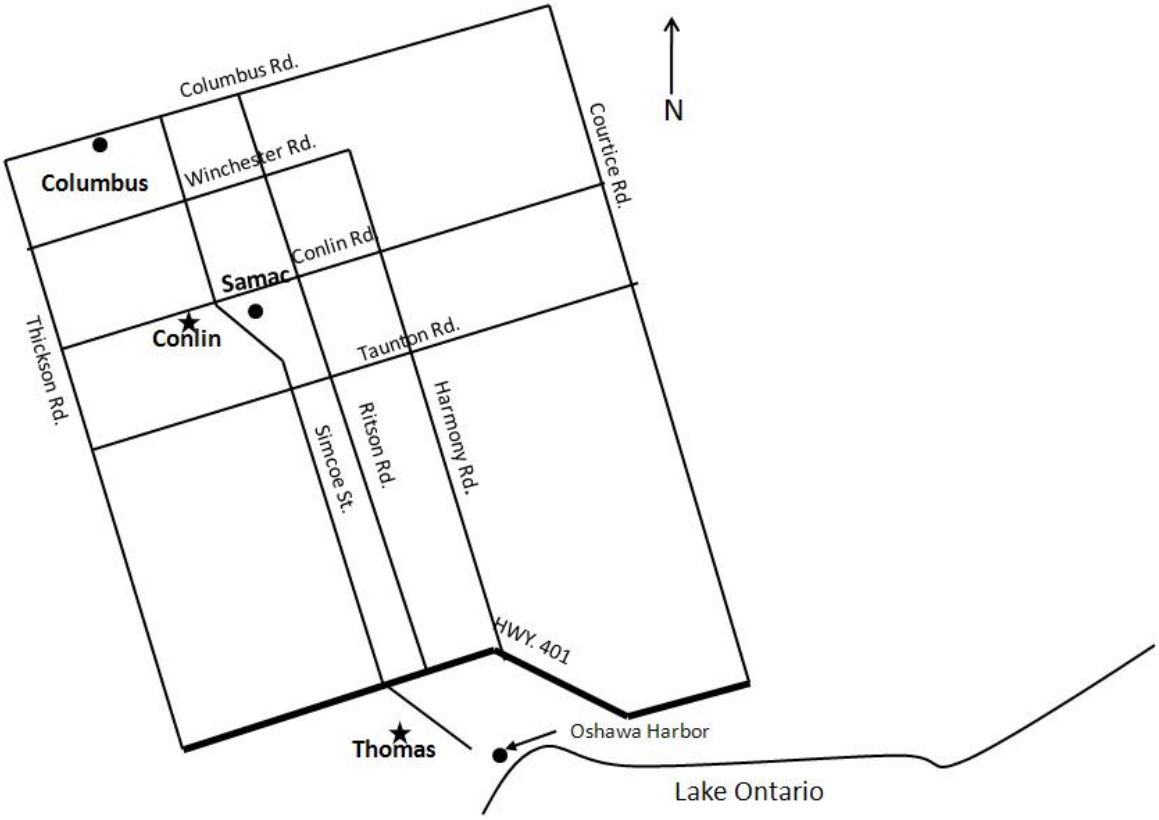

Figure 8.1: Locations of cages in the Oshawa Creek Watershed during the spring exposure. Starred placements indicate sites affected by TFM.

8.2.4 Field Exposure 2

The second field exposure was initiated on April 27th, 2009 by placing the cages in the river. Trout were place in the cages on May 4th, 2009 and the experiment was conducted as per section 7.2.4. The second field exposure concluded on May 29th, 2009 and fish were brought back to the lab and dissected as per 4.2.0.

8.2.5 Abiotic and Tissue Measurements

All abiotic and tissue measurements were conducted in the same manner as sections 7.2.5 and 7.2.6 except for dissolved oxygen and pH which were ascertained using an Oxy-check© and a Combo© (ph, EC, TDS, temperature waterproof tester) by Hanna Instruments . Dissolved oxygen was verified using the methodology described in section 7.2.5.2.

8.3.0 Results

All abiotic factors measured are detailed in **Table 8.1**. The only abiotic factor that was significantly different between sites was dissolved oxygen. Conlin was about 2 ppm higher in dissolved oxygen then all other sites.

Descriptive characteristics of the trout caged at the 4 sites can be found in **Table 8.2**. There was no difference in trout lengths, weights, condition factor, HSI or GSI in caged fish.

It should be noted that over the 25 day exposure in Oshawa Creek, 17 fish died and 2 escaped while putting the fish into the river. Of the 17 dead fish; 4 died at Columbus, 3 at Conlin, 2 at Thomas, and 8 at Samac. The trout lost at Samac all occurred after a large rain event which buried one cage under approximately 15 cm of sediment (killing 5). When putting the trout into their cages at the initiation of field exposure 2 escaped, one at Conlin and one at Thomas.

At the conclusion of the experiment there were a number of fish observed that had sores on their ventral sides.

Trout caged in Osahwa Creek were removed from the river 3 days early due to the Department of Fisheries and Oceans (DFO) applying a lampricide (3-Trifluoro-Methyl-4-Nitro-Phenol (TFM)) to Oshawa and Lynde Creek on May 20th, 2009. TFM was applied to 5 sites along Oshawa Creek, at Winchester Road, in Camp Samac (below dam), Conlin Road (2 sites), and a tributary to Oshawa Creek in Goodman Creek sub watershed. A total of 917.5 L of TFM was applied to Oshawa Creek at a concentration ranging from 6.0 to 7.8 ppm over a time period of 13 hours. Trout caged at Conlin and Thomas were exposed to the lampricide. However, there was no mortality at those sites following the one week post-exposure period.

As with the previous experiments, all trout biomarkers were tested for significant gender effects and if none were found data was pooled and analysis was continued. There were no significant differences in any biomarkers between genders. There were no significant differences in condition factor, HSI, GSI, liver EROD, plasma VTG, and brain AChE activity. However, there was a significant difference in liver CS and LDH activity.

Fish caged at Thomas and Samac experienced inhibited liver CS activity compared to fish caged at Columbus, Conlin, and time zero by approximately 1.5 – fold (**Figure 8.2**). However, fish at Samac and Thomas did not differ significantly in CS activity. Trout caged at Samac had the greatest inhibition of CS activity with an activity of 1.72 ± 0.13 μmol/min/mg protein. Liver LDH activity was significantly different for fish caged at Conlin and Thomas compared to trout caged at Columbus, Samac and time zero by approximately 2 – fold apiece (**Figure 8.3**). Fish housed at Conlin had the highest induction of liver LDH activity with a final activity level of 0.84 ± 0.16 μmol/min/mg protein.

Table 8.1: Descriptive abiotic factors for field sites on Oshawa Creek and off stream reference site (Camp Samac (East Branch)). Values are given as means ± standard deviation.

	Water flow (s/m)	Water Temperature (°C)	Dissolved oxygen (ppm)
Columbus	3.32 ± 0.56	13.15 ± 0.55	10.2 ± 0.15[a]
Conlin	2.14 ± 0.08	10.02 ± 3.68	12.93 ± 1.75
Thomas	2.49 ± 0.37	15.38 ± 1.49	10.47 ± 0.09[a]
Samac	3.55 ± 0.07	13.78 ± 1.24	10.45 ± 0.18[a]

	Alkalinity (ppm)	Hardness (ppm)	pH	Total Organic Content (ppt)
Columbus	300 ± 0	300 ± 0	8.37 ± 0.07	0.25 ± 0
Conlin	300 ± 0	300 ± 0	5.79 ± 2.62	0.28 ± 0
Thomas	300 ± 0	300 ± 0	8.28 ± 0.08	0.34 ± 0.01
Samac	300 ± 0	300 ± 0	8.39 ± 0.04	0.27 ± 0

*superscripts in common denote no significant difference.

Table 8.2: Descriptive rainbow trout characteristics for caged fish. Values are given as means ± standard deviation.

	n	Weight (g)	Standard Length (cm)	Condition Factor (g/cm^3)
Time zero	5	121.10 ± 5.76	20.04 ± 0.45	1.51 ± 0.06
Columbus	11	104.41 ± 4.65	19.74 ± 0.27	1.33 ± 0.03
Conlin	10	105.30 ± 12.88	20.86 ± 0.44	1.29 ± 0.07
Thomas	10	117.69 ± 9.49	20.41 ± 0.31	1.39 ± 0.13
Samac	17	121.94 ± 8.10	20.82 ± 0.42	1.34 ± 0.06

	Hepato somatic Index (%)	Gonado somatic Index (%)
Time zero	1.03 ± 0.044	0.086 ± 0.012
Columbus	1.22 ± 0.166	0.111 ± 0.023
Conlin	1.42 ± 0.129	0.121 ± 0.018
Thomas	0.987 ± 0.113	0.075 ± 0.013
Samac	1.318 ± 0.094	0.118 ± 0.028

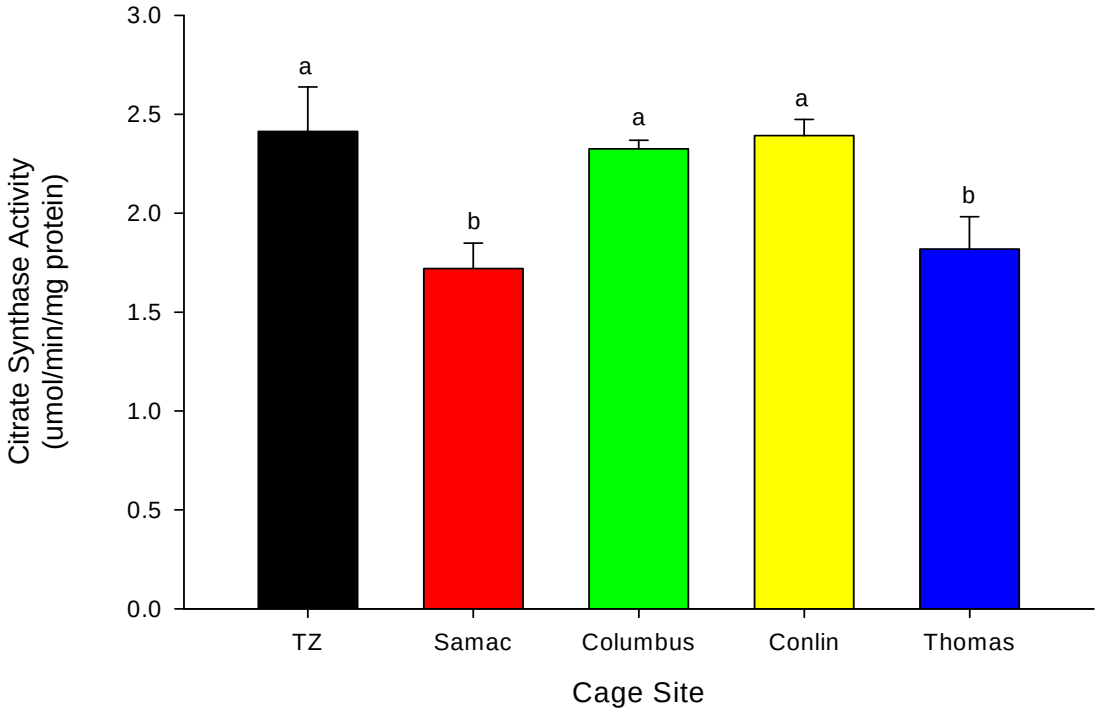

Figure 8.2: Liver CS activity of time zero fish (TZ) and trout caged in Oshawa Creek and the reference site, Camp Samac (East Branch). Values are given as means ± standard error. a indicates significance between time zero fish and those at Columbus and Conlin with fish caged at Samac and Thomas. b denotes that trout housed at Samac and Thomas were not significantly different to each other. Letters in common signify no significant difference.

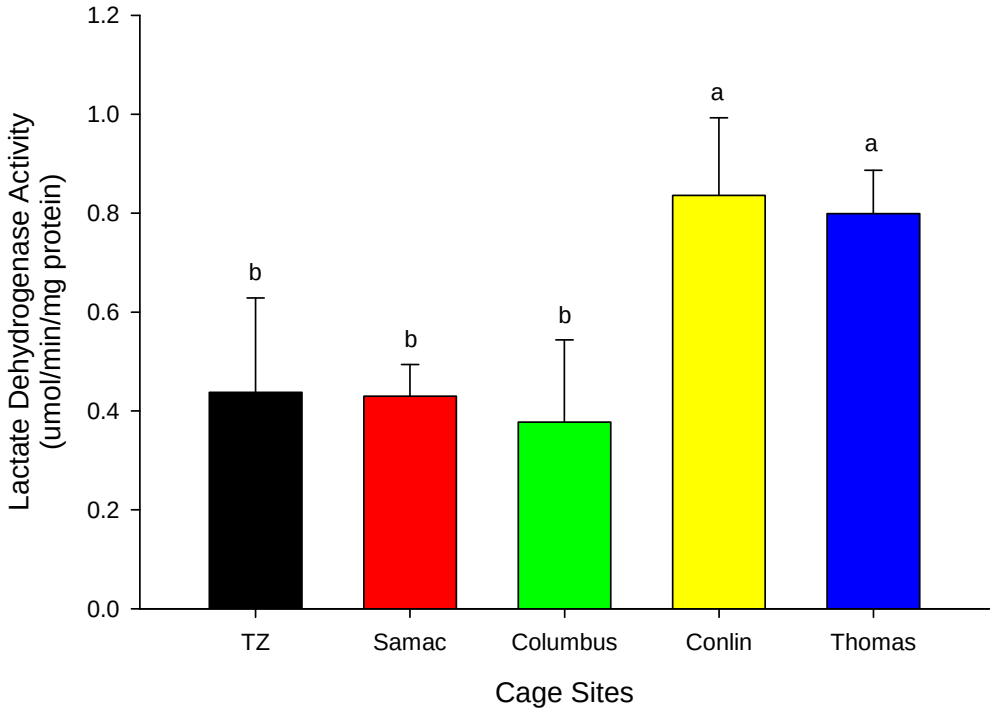

Figure 8.3: Liver LDH activity of time zero fish (TZ) and trout caged in Oshawa Creek and the reference site, Camp Samac (East Branch). Values are given as means ± standard error. a indicates significance between time zero fish and those at Samac and Columbus with fish caged at Conlin and Thomas. b denotes that trout housed at Samac, Columnus, and time zero fish were not significantly different to each other.

8.4.0 Discussion

This field exposure suffered higher mortality compared to the first one. The majority of the deaths occurred at the Samac site after a large rain event. Besides the loss of those fish the highest mortality rate was seen at the Columbus site. Unfortunately when the fish were discovered, they were too far decomposed to collect liver samples from to determine CS, LDH and EROD activity.

DO was significantly increased at Conlin compared to the other sites. DO levels were increased by about 2 ppm (DO at Conlin: 12.93 ± 1.75 ppm). Increased DO could represent increased stream health (little pollution). According to Snieszko (1974), aquatic environments exposed to higher levels of organic pollution have decreased DO levels. As observed in a river polluted by a cannery which had DO levels of 1.2 – 2.6 ppm (Snieszko, 1974). The high DO levels seen across all cage sites however, does not explain the increased mortality seen at Conlin.

There were significant changes in liver CS activity across sites with fish at Samac and Thomas having lower levels compared to time zero fish, and those at Columbus and Conlin. Fish at Samac would have experienced toxicants coming from agricultural, commercial, and residential lands just north of the site. Within the commercial area includes a golf course and it has been know for golf courses to use pesticides (i.e. diazinon) that are toxic to fish (Potter and Braman, 1991).

Fish at Thomas also experienced decreased CS activity. Fish at the Thomas site could have been affected from a number of sources (agricultural, commercial, industrial and residential) since this site is located almost at the end of Oshawa Creek near Lake Ontario. Without a specific point source it is impossible to tell what caused the CS inhibition in livers of fish at Thomas and Samac.

It is quite possible that decreased liver CS levels might have been observed in fish at Conlin too but, as previously mentioned; trout that had died during their time in the river were too decomposed to collect any tissue samples from.

Trout caged at Conlin and Thomas had significantly increased levels of liver LDH compared to the other sites. This is indicative of increased anaerobic metabolism (Battiprolu *et al.*, 2006). It would be expected that increased liver LDH activity would be seen in fish caged at Conlin and Thomas for two reasons. Fish caged at Conlin experienced the highest levels of mortality, possibly due to the agricultural runoff from the farm next door. Bagchi *et al.,* (1995) observed a similar increase in liver LDH activity with rats fed a variety of pesticides including; endrin, chlordane, alachlor, chlorpyrifos, and fenthion. This would be quite reasonable considering large amounts of pesticides are applied in the spring.

Secondly trout caged at Thomas were also downstream from all TFM application sites. TFM is a lampricide used to control parasitic sea lamprey (*Petromyzon marinus*) in the Great Lakes (Krueger and Spangler, 1980) has been shown to increase trout LDH levels significantly compared to the controls (Swift, 1978). Since there was no mortality at any of the sites treated with TFM it is reasonable to conclude that increased LDH activity would eventually return to baseline levels.

As previously mentioned Oshawa Creek was treated with the lampricide TFM (3-Trifluoro-Methyl-4-Nitro-Phenol) on May 20th, 2009. Two of the application points were upstream of the cages at Conlin and Thomas. Application at Conlin was approximately 100 m upstream from the cages. TFM has been known to be toxic to fish when under stressed conditions with the most sensitive being the channel catfish (96 h LC_{50} = 0.60 mg/L) and the least sensitive being the bluegill sunfish (96 h LC_{50} = 37 mg/L) (EPA, 1999). Other effects known to be caused by TFM include induced mixed function oxygenase activity, estrogen agonist, inducing VTG production and fish fry development abnormalities (EPA, 1999).

Since there were no significant differences in trout caged at any site for liver EROD, plasma VTG activity or fish mortality, it is safe to assume that TFM does not likely pose a risk to rainbow trout via pulse exposure.

8.5.0 Conclusion

From the spring field exposure in Oshawa Creek (west branch) and reference site Samac (east branch), it was noticeable that different biomarkers were affected, possibly due to different pesticides being used in the agricultural community. Oshawa Creek water affected both aerobic and anaerobic metabolism in exposed trout and TFM appeared to have no significant affects after a pulse exposure. Cage design still needs to be modified to prevent external injuries. Future exposures should be conducted to conclude the effects of contaminants in Oshawa Creek since the river was treated with TFM during the Spring. It would also be interesting to look at the east branch as well.

9. General Discussion

9.1.0 Laboratory Endosulfan Exposures

It was the aim of these studies to investigate the endocrine and metabolic disruption potential of endosulfan on rainbow trout. Rainbow trout were selected as a test organism because they are easily obtainable, do not stress easily from handling, and are sensitive to endosulfan through waterborne exposure (96 h LC_{50} = 1.7µg/L) (Capkin *et al.,* 2006). Furthermore, trout have already been proven to be a useful indicator species for endocrine and metabolic disruption as observed by Orrego *et al.,* (2009), Sherry *et al.,* (1999), and Battiprolu *et al.,* (2006). Consequently, due to these characteristics rainbow trout were chosen to investigate the effects of endosulfan. The fish were obtained from Rainbow Springs Trout Farm in Thamesford, ON.

At the initiation of this experiment there was little knowledge in the area of endosulfan being administered via i.p. injection in rainbow trout. There was also very little known about the metabolic and endocrine disruptive effects of endosulfan on rainbow trout via this route of exposure. There have been some studies indicating that endosulfan might act as an anti-estrogenic compound in rainbow trout (Harris *et al.,* 2000; Andersen *et al.,* 1997), along with indications that endosulfan might act through other pathways or act through a hormetic principal as observed in Japanese medaka by Gormley and Teather, (2003). Through two experiments where trout were exposed to endosulfan through a single i.p. injection (Sections 5 and 6), conflicting evidence of endocrine disruption was observed. In the first experiment there was large induction of plasma VTG activity in all treatments compared to the corn oil controls in fish sampled on day 2 followed by a reduction in VTG activity. In the second experiment there was very little plasma VTG activity in all treatments except the corn oil controls which had a large induction of VTG activity. Also found in the second experiment VTG activity in fish treated with 5 mg/kg 17-β estradiol had the same VTG activity compared to fish treated with the mix (**Figure 6.3.9**).

Differences in VTG activity between the two experiments could be due to the individual trout used. In the first experiment trout were larger (1-3 times larger) than those used in the second experiment. This size difference could have caused increased maturation in the trout, the sex ratio was approximately 1:1 males to females across all treatments which could explain the increased VTG activity across all treatments in those sampled on day 2. If all trout matured equally, there would be an equal expression in VTG activity which was seen in **Figure 5.3.6**. The second exposure, it was unsure why the corn oil controls had increased VTG activity compared to trout exposed to 17-β estradiol or the mix treatment. The increased corn oil control VTG activity is contradictory to what would be expected by that treatment as observed by Orrego *et al.,* (2009). The mix treatment was not different from the 0.32 mg/kg treatment, indicating that the concentration of endosulfan used in the mix was not concentrated enough to get a viable result indicative of endocrine disruption.

For future experiments to probe endocrine disruption with endosulfan it would be advised to use a triploid population to prevent biases in the data caused by gender. It would also be advised to use an increased number of mix treatments to ensure that the 17-β estradiol in the mix is not causing an equal VTG activity level as 17-β estradiol alone.

In both laboratory experiments, trout treated with endosulfan had inhibited liver CS activity compared to the corn oil controls. The decrease in liver CS activity is indicative of a reduction in aerobic metabolism (Kreb cycle) (Kuznetsov *et al.,* 2006). A reduction in CS activity can be caused by seasonal variation in water temperature as observed by St-Pierre *et al.,* (1998). However, trout were held at a constant temperature while in the lab thus, this is most likely not the situation. Tripathi and Verma (2004) also saw decreased liver CS activity in freshwater catfish which might be caused by reallocation of resources. It is possible that resources allocated for energy production, are being used to help detoxify endosulfan. If this were the case then it would have been expected to see increased liver EROD activity in both experiments. Liver EROD activity was only elevated in the first endosulfan experiment. Further testing should be conducted to ensure that endosulfan does induce EROD activity.

There was an increase in female liver LDH activity in both laboratory exposures which would be consistent with the observations of Tripathi and Verma (2004) of decreased Kreb cycle activity and increased gluconeogenesis. This enzyme was the only tissue biomarker assay that was significantly different due to gender. Female trout rely more heavily on anaerobic metabolism (Kuznetsov and Gnaiger, 2006) by approximately 30 % compared to males (Battiprolu *et al.,* 2006). In the second experiment there was less induction of LDH activity compared to the first exposure, this effect could be a result of the smaller trout used in the second exposure. Fish in the first experiment had livers that were approximately 1 to 1.5 – fold larger than the fish in the second experiment. The increased liver sizes would allow for a greater induction of LDH (Kurutaş *et al.,* 2006).

To generate more accurate results, it is recommended that similar sized triploid trout be used since they are all destined to be females. This would avoid differences between male and female liver CS and LDH activity. Also by looking at glucose – 6 – phosphate dehydrogenase, resource allocation can be further determined during endosulfan exposure.

9.2.0 Oshawa Creek Field Exposures

Through the course of the two field experiments (fall and spring), there was very little in common between the two seasons with respects to induction or inhibition of biomarkers. In the fall field exposure trout exhibited increased female liver EROD activity, decreased brain AChE activity, and significance in male HSI while, the spring field exposure only exhibited decreased liver CS activity and increased liver LDH activity. Oshawa Creek travels through several different demographics including: agricultural, industrial, commercial, and residential areas (CLOCA, 2002). The only demographic that would most likely have a seasonal variant in chemical contamination into Oshawa Creek would be from the agricultural sector. This would come from different pesticides or fertilizers being used for various pests at different times of the year.

The inhibited brain AChE experienced in the fall trial is representative of pesticide application, namely; carbamate and phosphate based pesticides (Sandhal and Jenkins, 2002; Siang *et al.,* 2007). Decreased brain AChE activity was seen in fish caged at Taunton and Thomas. Thomas is the farthest site south from the agricultural community, indicating that the toxicant in question might have come from another source. Unfortunately without a point source it is impossible to pinpoint the cause. The associated increase in liver EROD activity would be related to increase CYP 1A1 activity detoxifying the chemical causing the decreased brain AChE.

Even though the spring exposure in Oshawa Creek was supposed to be a temporal replicate of the fall exposure it is difficult to compare the two because the Department of Fisheries and Oceans treated Oshawa and Lynde Creek with a lampricide called TFM (3-Trifluoro-Methyl-4-Nitro-Phenol). The purpose of the lampricide is to eradicate larval and embryonic lampreys (EPA, 1997). Some of the application sites were approximately 100 m upstream from the cages and TFM has been known to cause mortality in fish that are stressed along with induced plasma VTG and liver EROD activity (EPA, 1997). Interestingly enough there was no induction of liver EROD or plasma VTG in fish from sites that got treated with TFM.

However, there was induction of liver LDH and inhibited liver CS activity indicating a change from aerobic to anaerobic metabolism as previously discussed (Tripathi and Verma, 2004). It would appear that TFM does cause some stress in trout as indicated by the opposing CS and LDH activities but, is not a significant threat to trout even at concentrated doses. It is possible that liver CS and LDH activity levels would have returned to normal if trout were left in Oshawa Creek for a longer period of time after TFM application.

9.3.0 Conclusion

From the laboratory and field experiments of rainbow trout it can be concluded that endosulfan does cause a disturbance in aerobic and anaerobic metabolism but its

potential as an endocrine disruptor needs further investigation. The use of triploid trout and more mix treatments are recommended. For the field experiments it would appear that fall exposure caused the largest induction of liver EROD activity and brain AChE inhibition. It is also apparent that application of TFM to a river does not pose a large risk to fish species inhabiting the treated watershed.

10. Summary

The Oshawa Creek watershed is a diverse system that flows from its spring fed headwaters north of Oshawa, to its outfall in Lake Ontario through the Oshawa Harbour. The creek flows through agricultural, residential, commercial and industrial areas before reaching Lake Ontario. It is reasonable to assume that Oshawa Creek may be exposed to a wide variety of toxicants from all the previously mentioned sectors. It is also safe to assume that on a temporal scale, the agricultural areas have the highest potential to release the most diverse amounts of toxicants into the Oshawa Creek watershed.

One of the potential toxicants that could be released is the pesticide endosulfan. Endosulfan is an organochlorine pesticide. Like most other organochlorine pesticides such as DDT, endosulfan was first introduced to North America in the 1950s as a safer alternative. Its use has spread across the world with some of the highest use still residing in North America, Durham region of Ontario. Endosulfan has been shown to be highly toxic to aquatic organism, especially fish. It is uncertain whether endosulfan has the potential to act as an endocrine disrupting chemical (anti estrogenic compound).

Rainbow trout (*Oncorhynchus mykiss*) were selected as a test species to preliminarily monitor Oshawa Creek and observe the effects of endosulfan on various biomarkers after a single i.p. injection. Rainbow trout are now native to Oshawa Creek watershed, easily obtainable, do not stress easily from handling, and have a wide variety of reliable biomarkers that can be tested. Biomarkers that were tested include; serum vitellogenin, liver ethoxyresorufin-O-deethylase, liver citrate synthase, liver lactate dehydrogenase, and brain acetylcholine esterase for trout caged in Oshawa Creek.

Trout exposed to 0.1, 0.32, 1.0, 3.2 mg/kg endosulfan and mix treatment (0.32 mg/kg endosulfan and 5 mg/kg 17-β estradiol) via a single i.p. injection, displayed induced female liver lactate dehydrogenase activity, along with inhibited liver citrate synthase activity during two different injection periods. In the first injection experiment

trout were observed to have increased ethoxyresorufin-O-deethylase activity and increased vitellogenin levels at sample day 2 unlike trout in the second experiment.

From these experiments it is apparent that endosulfan disrupts metabolic pathways in trout. However, confirmation as an endocrine disruptor (anti estrogenic compound) needs further experimentation possibly with the aid of more mix treatments.

Rainbow trout were caged at four locations along Oshawa Creek in the fall and spring of 2008-2009. The two exposures could not be counted as a full replication because the lampricide 3-Trifluoro-Methyl-4-Nitro-Phenol (TFM) was administered to Oshawa and Lynde Creek in the Spring of 2009, exposing half of the caged fish. However, there was increased mortality in the spring exposure compared to the fall in trout caged at Columbus. In the fall exposure, trout experienced increased liver ethoxyresorufin-O-deethylase activity in fish caged at Taunton and Conlin but no difference at the furthest site downstream (Thomas). Trout also experienced inhibited brain acetylcholine esterase activity at Taunton and Thomas indicating exposure to a carbamate or phosphate based pesticide.

Trout exposed to TFM did not have increased mortality, ethoxyresorufin-O-deethylase, or vitellogenin activity, but did have increased liver lactate dehydrogenase activity. TFM appears to cause slight metabolic disturbance in trout, but does not appear to pose a risk of mortality or liver impairment after a pulse exposure.

Based on the results of these experiments endosulfan needs further investigation as a potential endocrine disrupting chemical causing an anti-estrogenic effect in rainbow trout. Further characterization of Oshawa Creek is needed to account for seasonal variability in chemical contamination and the altered spring exposure due to Oshawa Creek being treated with TFM.

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