Embryotoxicity of dioxin-like chemicals extracted from American eels
(*Anguilla rostrata*) from the St. Lawrence River system

by

Sharilyn Judith Kennedy

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Abstract

The American eel (*Anguilla rostrata*) has suffered a serious population decline in Lake Ontario since the early 1980s due to a decline in recruitment of juveniles migrating from the Sargasso Sea. This has resulted in the closure of the Lake Ontario fishery in 2004 and its listing as endangered under the Ontario Species at Risk act in June of 2008 in Ontario. Due to their complex life cycle, little is known about eels once they leave their freshwater habitats and migrate to the Sargasso Sea to reproduce. Ocean conditions, habitat destruction, disease, reduced lipid content, over-fishing, physical barriers (hydroelectric dams), and chemical contamination are all possible reasons for recruitment decline and may be acting cumulatively. Maternally derived dioxin-like contaminants (DLCs) accumulated during the growth phase of eels in Lake Ontario are toxic to fish embryos, and embryotoxicity is expressed as a series of malformations known as blue sac disease (BSD). I assessed whether these toxicants are in high enough concentrations in sexually maturing eels to be embryotoxic to their offspring, as assessed by using Japanese medaka (*Oryzias latipes*), a surrogate species. Medaka embryos were first injected with 2,3,7,8-tetracholordibenzo-*p*-dioxin (TCDD) to establish their sensitivity to this test chemical expressed as an 11-day EC50 of 3.79 pg/mg, for the induction of BSD. Medaka embryos were injected with eel extracts and their response compared to the TCDD toxicity curve to assess whether extracts caused developmental problems and to estimate the relative concentration of DLCs. Eel extracts from all collection sites caused no dioxin-like toxicity to Japanese medaka embryos. However, significantly higher toxicity at 10 eeq relative to triolein was found for all extracts with no differences among sites, suggesting the presence of non-dioxin-like toxicants. The low level of maternal tissue contamination by DLCs implied by this bioassay is mirrored in chemical monitoring of persistent organic pollutants in Lake Ontario fish. If correct, the low levels of toxicity of extracts to embryos could contribute to the observed increase of eels entering L. Ontario from 2003 to 2008.
Co-Authorship

The following text was written by Sharilyn J. Kennedy, with significant contributions from my thesis supervisors, Peter V. Hodson, John Casselman, and Stephen Brown. Unless otherwise noted in the materials and methods, sample preparation and analyses were conducted by S.J. Kennedy. Statistical analyses and data interpretation were performed by S.J. Kennedy, in collaboration with P.V. Hodson, J. Casselman and S. Brown.
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<th>Description</th>
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<tr>
<td>AhR</td>
<td>aryl hydrocarbon receptor</td>
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<tr>
<td>BSD</td>
<td>blue sac disease</td>
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<td>CYP1A</td>
<td>cytochrome P450 1A</td>
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<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DLC</td>
<td>dioxin-like contaminant</td>
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<tr>
<td>EC50</td>
<td>median effective concentration</td>
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<tr>
<td>eeq</td>
<td>eel equivalent</td>
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<tr>
<td>FR</td>
<td>fin rot</td>
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<tr>
<td>GC/MS</td>
<td>gas column chromatography/ mass spectrometry</td>
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<tr>
<td>GPC</td>
<td>gel permeation chromatography</td>
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<tr>
<td>HAH</td>
<td>halogenated aromatic hydrocarbon</td>
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<tr>
<td>L.</td>
<td>Lake</td>
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<tr>
<td>LC50</td>
<td>median lethal concentration</td>
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<tr>
<td>MS-222</td>
<td>tricaine methanesulfonate</td>
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<tr>
<td>OE</td>
<td>ocular edema</td>
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<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
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<tr>
<td>PE</td>
<td>pericardial edema</td>
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<tr>
<td>PCB</td>
<td>Polychlorinated biphenyl</td>
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<tr>
<td>POP</td>
<td>persistent organic pollutant</td>
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<tr>
<td>R.</td>
<td>River</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SLR</td>
<td>St. Lawrence River</td>
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<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
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<tr>
<td>TEF</td>
<td>toxic equivalent factor</td>
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<tr>
<td>TEQ</td>
<td>toxic equivalent</td>
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<tr>
<td>TH</td>
<td>tube heart</td>
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<tr>
<td>YE</td>
<td>yolk sac edema</td>
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<tr>
<td>XRE</td>
<td>xenobiotic responsive elements</td>
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Chapter 1

General introduction and literature review

1.1 Declining Eel Abundance

1.1.1 Life History

The American eel (*Anguilla rostrata*) inhabits a broad range of environments including freshwater rivers, lakes, estuaries, coastal areas and the Atlantic Ocean from Greenland to Venezuela, making it one of the most geographically-dispersed fish species in the world (Shepherd, 2006). The life history of *A. rostrata* is complex and poorly known due to its far offshore migration and the fact that spawning has not yet been observed directly. Beginning and ending its life in the Sargasso Sea, *A. rostrata* is a catadromous species, spending most of its life in freshwater habitats before migrating back to the ocean to reproduce (Figure 1-1). There is strong evidence that suggests that each year, mature American eel from Greenland to Venezuela spawn together in the Sargasso Sea, resulting in only one true population; this is known as panmixia (Avise *et al*., 1986). *A. rostrata* is semelparous, breeding only once in its life and then dying where it was spawned. This may contribute to eels’ sensitivity to contamination because there is no offloading of body burdens during annual reproductive cycles as with iteroparous species. It is believed that mating occurs deep in the ocean between 2,000 m and 3,000 m in subtropical gyres (Tsukamoto *et al*., 2002). The subtropical gyre is crucial for the transport of both European and American eel species’ pelagic leptocephalus larvae (75-100 mm, 4-8 months old) to their growth habitats in Central and North America and Europe (Figure 1-2). The decreasing strength of the Gulf Stream by 30% over the past 12 years may seriously reduce migration success of the 5 cm larvae which drift with the current (Bryden *et al*., 2005).
Figure 1-1: Life cycle of *A. rostrata*, showing the location of various developmental stages. The dashed lines in the centre of the figure indicate that a freshwater life phase is not obligatory but optional for eels which stay in estuaries (Copied with permission from: Fisheries and Oceans Canada, 2006).
This will likely lengthen their transport time from the usual 4-8 months for American eels and 9-12 months for European eels to roughly 5-10 and 12-16 months respectively, increasing chances of predation before entering coastal waters.

Once in coastal waters, the larvae transform to transparent glass eels (5-8 cm) and migrate into estuaries where they become pigmented elvers (< 10 cm). Some elvers will remain in this brackish water (salinity of 0.5-17 ppt) while others will travel further upstream overcoming spillways, dams and falls; many will not survive this journey. Now in their yellow phase, eels will remain in their habitats for the majority of their lives, between 10-30 years, growing up to one and a half metres long at sexual maturity (Shepherd, 2006). This phase of the eels’ development is also known as the feeding stage, when they accumulate the majority of their contaminants (Knights, 1997; Daverat et al., 2006) and generally show low depuration rates (de Boer et al., 1994). Yellow eels are benthic omnivores, feeding on crustaceans, clams, worms, fish, frogs and dead animal matter. As eels grow, their feeding transforms into more aggressive piscivorous predatory behavior. With this capacity to eat larger prey, we see a progressive increase in trophic level and concentration of contaminants. As eels grow they also become higher in fat content; before beginning its migration back to the warm waters of the Sargasso Sea, A. rostrata must undergo one final physiological transformation into a silver eel. The eyes and pectoral fins enlarge, visual pigments change, the body colour transforms, muscle tissue is converted into gonad and the eel stops feeding. A. rostrata travel over 6,000 km in seven weeks from L. Ontario to the western part of the Sargasso Sea (Fisheries and Oceans Canada, 2006). The migration occurs throughout autumn nights for a synchronized February spawning coinciding with the new moon (Tsukamoto et al., 1998), and spawning may last for several months. Due to the mass aggregation in space and time, this spawning results in increased reproductive success.
Figure 1-2: Distribution and size of leptocephalus larvae of the European eel, *Anguilla anguilla* (solid line) and the American eel *Anguilla rostrata* (dashed line). Both originate at their common spawning ground in the Sargasso Sea. Isopleths represent the size of leptocephalus in mm (modified from Schmidt, 1923). The Sargasso sea is located roughly between 70° west to 40° west, and from 25° north to 35° north, bounded by the Gulfstream on the West, the Greater Antilles on the South, and Bermuda to the North.
1.1.2 Lake Ontario and the St. Lawrence River

The St. Lawrence River (SLR) flows for 760 km from Wolfe Island in Kingston, ON to Tadoussac, QC (Figure 1-3). At its outlet, it drains, on average, 14,165 m³/s of water and has a total drainage area of 1,030,000 km², making it one of the largest river systems in the world (Renwick, 2008). Due to its size, the SLR is also the most important commercial river in Canada, allowing for the passage of oceangoing vessels and linking the Great Lakes to the Atlantic Ocean. The Great Lakes basin has an increasingly dense population along its shores with eight states and two provinces sharing its banks. Approximately 15 million Canadians and 30 million Americans inhabit the basin, making the Great Lakes watershed one of the most densely populated and heavily contaminated areas in Canada (Renwick, 2008). Industries, specifically chemical and petroleum synthesis, steel manufacturing, aluminum smelters, paper mills, power generators and agriculture are the most important economic activities in the Great Lakes region. For decades these activities along with urban development, have contaminated the Great Lakes-St. Lawrence waterways (Lepage et al., 2000).

Polychlorinated biphenyl (PCB) levels in 62% of walleye, 83% of perch and 100% of sturgeons, exceeded the level recommended by the International Joint Commission (1.0 mg/kg) for the protection of aquatic biota in 2004 (Environment Canada, 2005). For a detailed explanation on individual contaminants to L. Ontario please refer to section 1.5.2. The decline of the eel population is just one of the many indicators of the poor environmental quality of the St. Lawrence River system as a whole.
Figure 1-3: Map of the St. Lawrence River drainage basin. The St. Lawrence River begins at Wolfe Island in Kingston ON, and flows into the Gulf of St. Lawrence at Tadoussac Quebec, the black bars indicate the start and end of the St. Lawrence River. (Castonguay et al., 1993).
1.1.3 Evolution and Oceanic Transport

Much of the information collected about American eels is extrapolated from its close relative, and better studied, European eel (*Anguilla anguilla*). For this reason it is important to give a brief explanation of the evolution of these two species to better understand their connection.

It is thought that 70 million years ago, during the Cretaceous-Eocene period, the oceanic ancestral eel split into three groups; one dispersed southwest to Australia and New Zealand, one went north to Japan, while the other moved along the northern margin of the Tethys Sea by larval transport in the Paleocircum global equatorial Current (Figure 1-4). Here the second group diverged into another two groups, one carried south to Africa and the other carried west to the Atlantic giving rise to the present day *A. rostrata* and *A. anguilla* (Tsukamoto et al., 2002). These two eels are thus closely related with only slight physical differences (greater number of vertebrae in *A. anguilla*). The primary factors that separate the American eel from breeding with the European eel appear to be a two month time difference in peak spawning (February and April respectively), and species-specific pheromone recognition (McCleave, 1987).

However, Avise *et al.* (1990) and Maes (2005) separately observed hybrids between both species in Iceland, indicating an intermediate migration loop and developmental time. The mechanisms by which the two species of eels diverge to their respective continents are unclear. Kleckner and McCleave (1985) proposed the possibility of a genetically-determined active choice of the water currents based on olfactory cues used by the larvae. Another theory is of a strict, genetically-determined period of metamorphosis which times the larval development perfectly with the distance to appropriate currents (Cheng and Tzeng, 1996). Larvae less than 5 mm in length drift with the prevailing currents. Cheng and Tzeng’s (1996) hypothesis implies that European eels develop more slowly than American eels, drifting for longer in the Gulf Stream, and disembarking later than its faster developing cousin.
Figure 1-4: Migration and speciation of Anguillid eels. The star indicates the proposed origin of the ancestral eel in Indonesia; the arrows indicate the direction of migration as well as the phylogeny. *A. rostrata* and *A. anguilla* are more closely related to each other than to any other eel species (Tsukamoto *et al.*, 2002).
1.2 Population Status

Since 1982, recruitment of *A. rostrata* to L. Ontario has diminished dramatically with more than a 99% decline in the number climbing the eel ladder at Cornwall, the only index of recruitment until 2005 (OMNR, 2009). Because of this population crash, the L. Ontario eel fishery closed in 2004 and the eels in L. Ontario were listed as endangered under the Ontario Species at Risk Act in June 2008. However, this problem is not unique to the SLR. The Japanese eel (*Anguilla japonica*) has declined by 99% in the last three and a half decades, and the European eel by 90% in the last two decades (Dekker, 2003). Because all *A. rostrata* from Canada, the USA, and Central America breed together at a common spawning site (panmixia), the decline of eels in Canada could indicate a severe species-wide decline in recruitment (Casselman, 2003).

Additionally, the mechanisms that determine sex differentiation are poorly understood; it has been suggested that water temperature (Holmgren, 1996) and density of eels (Krueger and Oliveira, 1999) may play a role. It is also unclear whether habitat determines the sex of undifferentiated eels or if the already sexually determined eels select a particular habitat. What is important to note however, is that Castonguay et al. (1994a) reported that eels in the SLR were almost exclusively female, and were estimated to contribute about 50% of the female breeding population in the Sargasso Sea. Therefore, prior to 1984, L. Ontario contributed a significant proportion of gametes to the breeding pool, making recruitment decline of the SLR species an even bigger crisis affecting the entire species (Casselman 2003).

Construction of the Beauharnois dam in Beauharnois, QC began in 1929 and was completed in 1961. Two eel ladders, along with electronic eel counters, were installed at either end of the dam in 2002 and 2004 (Bernard and Desrocher, 2007). The earliest and best documented index of the declining species comes from the yellow eels ascending the eel ladder at the Moses Saunders Hydroelectric Dam in Cornwall, Ontario (Figure 1-5). All eels ascending this ladder had to overcome the downstream Beauharnois dam. The eel index however, is not free from bias. When the eel ladder was finally built in 1974, the eels below the dam had been accumulating for several years, allowing many years worth of
migrating eels to pass. This may have made the first few years of data collected on ascending eels artificially high. Early problems with the eel counter also became evident, as well as double counting of eels that got sucked back down the turbine and then re-climbed the ladder (Cairns et al., 2008). Construction of the eel ladder on the American side (Moses) of the dam in 2005 added more uncertainty to the index due to increased numbers of eels ascending that ladder compared to the ladder on the Canadian side (Saunders). This could be due to turbine speed or construction material of the ladder itself. It must be assumed that the eel index for the Saunders ladder, prior to the addition of the Moses ladder, is equal to the sum of the eels climbing both ladders. All of these problems were taken into account by Casselman (2003), who determined that the eel decline was real despite all of the biases of the index. Because the ladder provides the only recruitment index of the eel population, there are no data to conduct comparisons among other rivers in North America (Castonguay et al., 1994b).

Although far from all being identified, possible anthropogenic and natural causes thus far are summarized into two groups: Oceanic modifications: weakening of the Gulf Stream due to ocean warming, and increased freshwater input from melting icecaps which changes the migration route of leptocephali larvae (Castonguay et al., 1994b). Glass eel recruitment is closely correlated with the North Atlantic Oscillation (NAO), which can alter the position of the Gulf Stream (Knights, 2003).

    *Freshwater modifications:* habitat destruction, river regulation, chemical contamination, physical barriers to migration (both up stream and down; Casselman, 2003), over harvesting, and infection by introduced swim bladder nematode (Anguillicola crassus; Nimeth et al., 2000). However, it is more commonly believed that the continental factors are acting together to produce the exponential reduction in recruitment. My research will focus on chemical contamination, specifically dioxin-like contaminants (DLCs), and how this affects embryo-toxicity and recruitment failure to L. Ontario.

There is a possible case for contaminants in L. Ontario due to the failed recovery of lake trout (Salvelinus namaycush) to this area. Lake trout have experienced similar population declines as eels.
Figure 1-5: The number of juvenile eels climbing the eel ladders (a series of stainless steel, switch-back troughs, lined with artificial grass for traction) at the Moses (American)-Saunders (Canadian) Hydroelectric dam Cornwall, ON (OMNR, 2010).
In the early 1980s the lake trout population failed to recover from lamprey predation and over-fishing despite efforts from both Canadian and American fishery agencies to improve water quality, reduce lamprey attacks, and restrict trout fisheries. Due to the coincidence of the trout disappearance with the history of anthropogenic chemicals discharged into water, sediments, and biota of the Great Lakes basin, a link to toxicity was drawn and is one hypothesis about the decline of lake trout populations (Cook et al., 2003).

1.3 Contamination

Persistent lipophilic contaminants such as halogenated aromatic hydrocarbons (HAH) including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) are widespread environmental contaminants of air, water, sediments, wildlife, humans and fish (Safe, 1989). Halogenated aromatics are a highly stable class of chemicals, meaning they do not degrade easily, in contrast to other common chemicals such as ammonia. For example, the half life of ammonia in soil is 2.2 years, whereas the half life of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in soil is 12 years (Butler et al., 2003; Young, 1984). HAHs are present in diverse analytes as highly complex mixtures of isomers and congeners, greatly complicating the hazard and risk assessment of these compounds (Safe, 1990). The most toxic HAH is 2,3,7,8-TCDD (Van den Berg et al., 1998), which has been established as a bench mark for determining the relative toxicities of individual DLCs (i.e., toxic equivalents). A contaminant is “dioxin-like” if it is an isostereomer (similar in structure) of 2,3,7,8-TCDD, and thus has similar toxic effects because of its aryl hydrocarbon receptor (AhR) binding affinity (Metcalf and Metcalf, 1997). Table 1-1 summarizes the proposed TEF values for the major classes of TCDD-like HAHs, which are closely related to their structure and degree of chlorination, four or five chlorines being the most toxic (Safe, 1987).

Due to its mostly planar configuration (Figure 1-6), TCDD easily fits into the AhR, a protein receptor which acts as a transcription factor initiating a signaling pathway for the synthesis of some of the cytochrome P450 proteins.
Table 1-1. Summary of World Health Organization suggested Toxic Equivalency Factors (TEFs) found in humans, mammals, fish and birds for some important PCDDs, PCDFs and PCBs (Van den Berg et al., 1998).

<table>
<thead>
<tr>
<th>Congener</th>
<th>Humans/mammals</th>
<th>Fish&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Birds&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,7,8-TCDD</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1,2,3,7,8-PentaCDD</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,2,3,4,7,8-HexaCDD</td>
<td>0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5</td>
<td>0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,2,3,6,7,8-HexaCDD</td>
<td>0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
<td>0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,2,3,7,8,9-HexaCDD</td>
<td>0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,2,3,4,6,7,8-HeptaCDD</td>
<td>0.01</td>
<td>0.001</td>
<td>&lt;0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>OctaCDD</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>2,3,7,8-TetraCDF</td>
<td>0.1</td>
<td>0.05</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,2,3,7,8-PentaCDF</td>
<td>0.05</td>
<td>0.05</td>
<td>0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2,3,4,7,8-PentaCDF</td>
<td>0.5</td>
<td>0.5</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,2,3,4,7,8-HexaCDF</td>
<td>0.1</td>
<td>0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.1&lt;sup&gt;b,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,2,3,6,7,8-HexaCDF</td>
<td>0.1</td>
<td>0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.1&lt;sup&gt;b,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,2,3,7,8,9-HexaCDF</td>
<td>0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>0.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>2,3,4,6,7,8-HexaCDF</td>
<td>0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>0.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,2,3,4,6,7,8-HeptaCDF</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,2,3,4,7,8,9-HeptaCDF</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;c,e&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>OctaCDF</td>
<td>0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0001&lt;sup&gt;c,a&lt;/sup&gt;</td>
<td>0.0001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>3,4,4',5-TetraCB (81)</td>
<td>0.0001&lt;sup&gt;a,b,c,d&lt;/sup&gt;</td>
<td>0.0005</td>
<td>0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3,3',4,4'-TetraCB (77)</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.05</td>
</tr>
<tr>
<td>3,3',4,4',5-PentaCB (126)</td>
<td>0.1</td>
<td>0.005</td>
<td>0.1</td>
</tr>
<tr>
<td>3,3',4,4',5,5'-HexaCB (169)</td>
<td>0.01</td>
<td>0.00005</td>
<td>0.001</td>
</tr>
<tr>
<td>2,3,3',4,4'-PentaCB (105)</td>
<td>0.0001</td>
<td>&lt;0.000005</td>
<td>0.0001</td>
</tr>
<tr>
<td>2,3,4,4',5-PentaCB (114)</td>
<td>0.0005&lt;sup&gt;a,d,e,f&lt;/sup&gt;</td>
<td>&lt;0.000005&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.0001&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>2,3,4,4',5-PentCB (118)</td>
<td>0.0001</td>
<td>&lt;0.000005</td>
<td>0.0001</td>
</tr>
<tr>
<td>2,3,3',4,4',5-PentaCB (123)</td>
<td>0.0001&lt;sup&gt;c,d,f&lt;/sup&gt;</td>
<td>&lt;0.000005&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.0001&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>2,3,3',4,4',5-HexaCB (156)</td>
<td>0.0005&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>&lt;0.000005</td>
<td>0.0001</td>
</tr>
<tr>
<td>2,3,3',4,4',5-HexaCB (157)</td>
<td>0.0005&lt;sup&gt;d,e,f&lt;/sup&gt;</td>
<td>&lt;0.000005&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>0.0001</td>
</tr>
<tr>
<td>2,3,4,4',5,5'-HexaCB (167)</td>
<td>0.00001&lt;sup&gt;a,f&lt;/sup&gt;</td>
<td>&lt;0.000000&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.0001&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>2,3,3',5,5'-HeptaCB (189)</td>
<td>0.0001&lt;sup&gt;c,a,d&lt;/sup&gt;</td>
<td>&lt;0.000005&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.0001&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviations: CDD, chlorinated dibenzodioxins; CDF, chlorinated denzo furans; CB, chlorinated biphenyls; QSAR, quantitative structure-activity relationship.

<sup>a</sup>Limited data set.
<sup>b</sup>in vivo CYP1A induction after in ovo exposure.
<sup>c</sup>in vitro CYP1A induction.
<sup>d</sup>QSAR modeling prediction from CYP1A induction (monkey, pig, chicken, or fish).
<sup>e</sup>Structural similarity.
<sup>f</sup>No new data from 1993 review (1).
<sup>g</sup>QSAR modeling prediction from class specific TEFs.
Figure 1-6: 2,3,7,8-TCDD molecule with ordered numbering on binding sites, and β and α positions labeled.
Exposure of fish embryos to this chemical can greatly impair development, cause blue sac disease (BSD), and increase mortality rate. Early life stages of fish are more sensitive than adults to the effects of these chemicals because exposure is occurring during critical developmental periods (Walker and Peterson, 1994). This can result in a reduction of recruitment. Most HAH contamination is anthropogenic, and the major sources are: combustion of PVC piping (or any organic materials that contains chlorine), production of herbicides and fungicides (e.g. 2,4-dichlorophenol, 2,4,5-trichlorophenol and chlorophenoxy acetic acid or agent orange), pulp and paper production (which releases furans as well), and industrial chemical production (e.g. Dow Chemical Co.’s production of chlorophenols); (Health Canada, 2001). Once in the water, these hydrophobic chemicals can adsorb to particulate organic matter, and precipitate into sediments where they begin accumulating up the food chain. As well, fish can be exposed by contact of respiratory surfaces with contaminated water, permitting direct chemical uptake through the gills. As previously mentioned, A. rostrata are long-lived benthic top predators with a high fat content, which makes them particularly susceptible to accumulating high loads of lipophilic (hydrophobic) toxicants (Couillard et al., 1997).

Before migration back to the Sargasso Sea, sexually maturing eels stop eating and their gonads begin to mature. The fat stores of the body, which also act as stores of DLCs, are transferred from the muscle to the developing reproductive organs, resulting in dioxin concentrations which are likely higher in the ovaries than any other tissue because ovaries contain such a high lipid content (Couillard et al., 1997). Cook et al. (2003) found that elimination of TCDD and related chemicals by lake trout embryos and sac fry is slow, so that almost the entire maternal load transferred to the offspring is retained during embryonic development. I propose that this is probably the case for A. rostrata as well, although the embryo developmental time for lake trout is much slower than for eels. This assumption is critical to the maternal transfer hypothesis of this study. Thus, injected concentrations of eel extracts in medaka eggs were used to model overall early life stage exposure.

The effects of halogenated hydrocarbon (HAH) exposure are mediated by the AhR, a transcription factor that brings about changes in gene expression, inducing cytochrome P4501A (CYP1A)
enzymes, and affecting toxicity of HAH (Hankinson, 1995), the details of which are described in section 1.4.1 The AhR protein plays a critical role in development, adaptation to hypoxia, metabolism of aromatic compounds, and modulation of growth factor signaling, making its untimely activation very dangerous in developing organisms (Schmidt and Bradfield, 1996). HAH toxicity can be predicted by its AhR-binding affinity and CYP1A induction potency (Denison and Heath-Pagliuso, 1998). CYP1A induction has been used to illustrate altered gene expression following TCDD exposure (Denison and Heath-Pagliuso, 1998).

There are two isoforms of the AhR in fish, AhR1 and AhR2, that DLCs can bind to and increase the expression of CYP1A (Hahn, 1998). AhR is a member of the basic Helix-loop-helix-Per-ARNT-sim (bHLH-PAS) family of ligand activated transcriptional regulators. The AhR controls a variety of developmental and physiological activities, including neurogenesis, tracheal and salivary duct formation, chemical metabolism, circadian rhythms, response to hypoxia, cardiac development, and hormone receptor function (Bock and Kohle, 2006). The AhR is known to interact with signaling pathways that are mediated by estrogen, androgen and thyroid hormone receptors. It can result in decreased numbers of estrogen receptors and an increase in estrogen metabolism (Hestermann and Brown, 2003). Activation of the AhR has also been linked to a reduced stress response in fish. The AhR represses the corticosteroid pathway in the kidney of adult fish, preventing the fish from reacting to traumatic situations (Aluru and Vijayan, 2006). The implications of this to juvenile fish have not yet been established.

1.3.1 The Family of Chemicals

Aromatic hydrocarbons (AHs) are a class of organic chemicals which are lipophilic and carcinogenic. They were so named because of their sweet scent, but aromaticity also refers to a chemical property by which a planar, cyclic, conjugated benzene ring exhibits stabilization stronger than would be expected by the stabilization of conjugation alone (Schleyer, 2001). AHs can be subdivided into two groups; mono aromatics and polyaromatics. Polyanromatic hydrocarbons (PAHs) are ubiquitous in the environment as well as persistent and bioaccumulative. PAHs typically have half lives between 2-10 years; e.g. 2,3,7,8-TCDD has a half life in the environment of 10-12 years and in human serum of 7.5
years (Young, 1981). This indicates that contaminants can be persistent but the degree of persistence depends on the medium they are in. Members of the PAH family include the PCDD/PCDFs, PCBs, PCDEs, as well as the polybrominated biphenyls (PBBs) and polybrominated diphenyl ethers (PBDEs). Each subfamily consists of many congeners that share the subfamily's chemical backbone but with different numbers and positions of halogen substituents. Polyaromatics can be divided again into two subgroups; non-halogenated and halogenated AHs (Figure 1-7). As previously mentioned, the most toxic and persistent of these AhR-agonists is the halogenated 2,3,7,8-TCDD, a lipophilic compound found rarely in high concentrations in nature.

1.3.2 Dioxins/Furans (TCDD/F)

TCDD/Fs, like all aromatic hydrocarbons, are persistent, lipophilic, bioaccumulative and biomagnify through the food chain. TCDD was first recognized as a serious problem of the Great Lakes in the 1960s when it was discovered in high concentrations in the eggs and tissues of top predatory animals such as lake trout and herring gulls (Cook et al., 2003). Cook et al., (2003), noticed that there was a correlation in the peak production of DLCs and the peak in contaminants found in eggs and sediment (Figure 1-8). He concluded that the predicted sac fry mortality due to AhR-mediated toxicity alone could explain the extirpation of lake trout from L. Ontario. Although TCDD production and disposal is heavily regulated in Canada and the U.S., this extremely stable toxicant still remains in biota.

TCDD/Fs are an inadvertent by-product of various processes. Currently the major contributor of dioxins and furans to the L. Ontario ecosystem is Aleris Specification Alloy Products Canada Company in Mississauga, Ontario. Through its production of molten primary metals, Aleris releases 0.72 g TEQ/year into the air and disposes of 13 g TEQ/year off site (NPRI, 2006). To a lesser extent, forest fires, wood burning stoves, and internal combustion engines are also sources of dioxins/furans, contributing approximately 5 g/year to the lake through atmospheric deposition (Glutting, 2003). Because L. Ontario is the eastern-most lake and the lowest in elevation of the Great Lakes, it receives most of its water from the
preceding four lakes through the Niagara River. This water passes through one of the most industrialized and heavily populated areas of Canada and the United States (Shen et al., 2008).

**Figure 1-7:** The family of chemicals that fall under the umbrella of aromatic hydrocarbons. The DLCs of concern (in bold) are the halogenated polyaromatics and some PAHs which cause AhR mediated toxicity to early life stages of fish.
Figure 1-8: Retrospectively determined toxic equivalent concentrations in eggs (TEC_{eggs}) from analysis of sediment cores in south central Lake Ontario. The concentrations of each AhR agonist was estimated from the concentrations of organic carbon in the sediment and lipid concentrations in eggs to estimate each chemical’s contribution to the TEC_{eggs}. Predicted TEC_{eggs} are compared to TEC_{eggs} measured in lake trout and estimated from TCDD concentrations measured in herring gull eggs (Cook et al., 2003).
Many manufacturing and processing plants along with substantial amounts of agricultural land are located on the shores of the Great Lakes and its tributaries. The lake wide average TEQ for PCDD/Fs in sediments was 101 pg/g in 2002, greatly exceeding the probable effects level guidelines of 21.5 pg/g of PCDD/F (Marvin et al., 2002). The distribution of PCDD/Fs in L. Ontario sediments may be related to current circulation. Beletsky et al. (1999) observed that L. Ontario has a predominant circulation throughout its three main basins (Niagara, Mississauga, and Rochester) which results in a more even distribution of contaminants and a gradient of concentrations from low, at the shallow near-shore sediments, to more concentrated at the deep-water depositional zone sediments.

Primitive organisms such as amphipods are less capable of metabolizing DLCs and so they biomagnify at these lower trophic levels. Lipophilic contaminants build up in the fatty tissues of organisms and bioaccumulate up the food chain where they contaminate top predators such as piscivorous fish, birds and mammals (Figure 1-9). PCDFs are typically metabolized more rapidly than PCDDs (Parrott et al., 1995). Although Figure 1-9 is a good illustration of the contaminant levels in the food web of L. Ontario, it is not without bias. The plankton data may be elevated due to the high lipid content of some species. Similarly, the low lipid content of trout reduces its TEQ value compared to that of eels. The eel data included in this figure were collected from eels between 18 and 30 years of age, however the trout data were obtained from fish that were about 6 years old. Also, these data may not reflect current contaminant levels considering the year of data collection, especially eels that have declined in contaminant concentration by a factor of 1000 since 1992. Despite these limitations, it is evident that DLCs are bioaccumulating up the L. Ontario food chain and that eels are accumulating a substantial load.

Dioxins and furans are very toxic, carcinogenic, and teratogenic. They may also cause critical endocrine disruption (Cook et al., 1994). There are 75 possible congeners of TCDD, only seven of which are listed as priority toxicants due to their structure and persistence. The congeners differ in the number of chlorines, ranging from one to eight. The toxic potency of these congeners and those of PCDFs, and PCBs, depends on the number of chlorines and the substitution pattern.
Figure 1-9: Toxic Equivalents (TEQs) for PCB congeners in water, sediment and biota from the L. Ontario food web (modified from Metcalfe and Metcalfe 1997). Eel data were obtained from Hodson et al., (1994). Abbreviations are: Sed., Sediment; Plank, Plankton; and Alwf., Alewife.
Some congeners have chlorine substitution patterns that create molecular geometries with planar configurations similar to that of 2,3,7,8-TCDD (Cook et al., 2003). The greatest toxicity is characterized by a molecule with four or five chlorines bound to the β positions as opposed to the α positions (Figure 1-6). Both of these factors contribute to the overall shape of the congener. A congener like 2,3,7,8-TCDD has a molecular size and conformation that allows it to bind easily to the AhR, i.e., it and other similarly shaped congeners are potent AhR agonists. In contrast, a highly chlorinated congener like octochlorodibenzo-p-dioxin cannot fit the AhR as easily and is thus less toxic.

1.3.3 Non-dioxin/furan Contaminants

The following toxicants are listed as lake-wide management plan critical pollutants because levels of these contaminants in L. Ontario fish and wildlife continue to exceed human health standards.

1.3.3.1 PCBs

Polychlorinated biphenyls (PCBs) were widely used as electrical insulators and flame retardants in transformers and as industrial lubricants between 1929 and 1978. The U.S. alone manufactured 1.4 billion pounds, 0.9 billion of which Canada imported before manufacturing bans were implemented (Glutting, 2003). PCB concentrations peaked in L. Ontario around 1985, and similar patterns were found in fish and in sediments (Cook, 2003). The hazards posed by PCBs were discovered in the 1960s when they were found to bioaccumulate to levels of concern in a wide range of organisms. These organisms, specifically top predators like lake trout, experienced reproductive failure. PCBs behave in a similar fashion to dioxins in that their toxicity is mediated by the very pathway that metabolises them, the AhR pathway.

In North America, most PCBs were commercially produced as standard mixtures referred to by their brand name, Aroclor. Aroclors are typically made of 110-120 PCB congeners, so that each Aroclor has a unique signature based on its congener composition. There are twelve dioxin-like PCB congeners, of which PCB126 is the most toxic due to its similar shape to 2,3,7,8-TCDD. Once in the environment, the composition of the Aroclors is unstable due to weathering of the mixtures (Erickson, 1997). An
environmental sample can be difficult to match to an Aroclor pattern once weathering has occurred. This can lead to difficulties in identification and quantification of PCBs. Another way of monitoring PCBs in the environment involves quantifying the concentrations of individual congeners in PCB-contaminated media. This procedure provides a more accurate estimate of total PCB concentrations, regardless of the extent of weathering or number of Aroclors present. More recently, the focus has been placed on the 12 aforementioned PCB congeners for which toxicity is similar to that of 2,3,7,8- TCDD, and other chlorinated dioxins/furans (Safe, 1992; ). By estimating dioxin toxicity equivalence (TEQ) for dioxin-like congeners, assessors can evaluate the risk associated with these congeners separately from total PCBs.

The majority of PCBs entering L. Ontario come from the upstream Great Lakes basins, contributing 302 kg/year. Within the L. Ontario basin almost 100 kg/year of PCBs are deposited by point (wastewater treatment facilities) and non-point sources, 80% of which enters the lake via streams and rivers. Atmospheric loadings are also a considerable source of PCBs to the lake; contributing 64 kg/year directly to the lake’s surface (Glutting, 2003). However, PCBs are volatile and the lake loses about 440 kg/year through evaporation. When this loss is considered, the total amount of PCBs within L. Ontario appears to be decreasing at a rate of 250 kg/year (Glutting, 2003). The remaining PCBs are transferred downstream or buried in the sediments.

1.3.3.2 PBDEs

In 1997 a study conducted by Luross et al. (2002) found that L. Ontario lake trout had the highest concentration of polybrominated diphenyl ethers (PBDEs) of any of the Great Lakes, with 95 ± 22 ng/g ww. In all the L. Ontario samples, the predominant PBDE congeners (2, 2’, 4, 4’-tetrabromodiphenyl ether (BDE-47), 2, 2’, 4, 4’, 5-pentabromodiphenyl ether (BDE-99), and 2, 2’, 4, 4’, 6-pentabromodiphenyl ether (BDE-100)), make up the primary components of the commonly used penta-BDE formulation flame retardant. PBDE congeners exhibit similar AhR mediated effects and potencies to PCBs but are more susceptible to degradation because bromine is more easily removed than chlorine
(Hooper, 2000). However, the degraded congeners of PBDEs are readily bioaccumulated, some almost as well as PCBs, and the environmental fate of these molecules are not well documented (Hooper, 2000).

1.3.3.3 PCDEs

Polychlorinated diphenyl ethers (PCDEs) are HAHs that have structural similarities to PCBEs and TCDD/F. They are lipophilic, resistant to degradation, and bioaccumulate. PCDEs have been detected in piscivorous fish, birds and humans, although its AhR potency is quite low (Koistinen, 2000). On the other hand, PCDEs are structurally similar to thyroid hormone and could thus bind to thyroid hormone receptors altering their function. Furthermore, PCDEs might be metabolized in the environment to toxic congeners or photolyzed to PCDDs and PCDFs (Koistinen, 2000). Metcalf et al. (1997) exposed Japanese medaka (Oryzias latipes) to extracts of L. Ontario Lake trout and found the LC50 for embryotoxicity to be equivalent to 15.5 ng/g of total PCDEs. This indicates that PCDEs in lake trout have the potential to induce toxic effects in early life stages of fish. Additionally, the mean total PCDE concentrations in L. Ontario biota can be as high as 672 ng/g ww in white sucker (Catostomus commersonii) and 791 ng/g ww in brown bullheads (Ameiurus nebulosus) (Villeneuve et al., 1999), demonstrating that PCDE levels are high enough to cause embryotoxicity in Great Lakes fish.

The actual amount of persistent organic pollutants (POPs) maternally transferred to an eel embryo will depend on the physiology of migration and sexual maturation. Because POPs are stored in lipids, they are likely at equilibrium throughout the body, and there should be no difference in POP concentrations per mg of lipid between parent tissues and ovaries, as observed by Hodson et al. (1994). However, the concentrations of POPs may increase during sexual maturation and migration. Because eels stop feeding prior to their spawning migrating, a reduction in overall lipid volume would be anticipated as lipids are converted to energy. An alternative possibility is that migrating eels conserve their lipid stores for egg development by converting protein from unneeded tissues (e.g., the gut) to energy. In this case, POPs stored in the lipid would not increase substantially during migration.
1.4 Mechanisms of Toxicity

1.4.1 Aryl Hydrocarbon Receptor (AhR)

Once ingested, the body tries to metabolize and eliminate DLCs by making them more water soluble. In the case of TCDD, and similarly shaped molecules, the primary mechanism of toxicity is through a common receptor mediated binding of the AhR, also known as the dioxin receptor. Metabolism of dioxins, furans and PCBs usually takes place in liver cells where the xenobiotic “TCDD-like” ligand can easily cross the cell membrane, entering the cytoplasm and binding to the AhR (1 in Figure 1-10). While it is bound to two heat shock proteins and an AhR inhibitory protein, the AhR exists in a dormant state in the cytosol (Hahn, 1998). Upon ligand binding, the complex dissociates with the heat shock and inhibitory proteins transporting TCDD into the nucleus (2 and 3 in Figure 1-10) where it forms a heterodimer with an aryl hydrocarbon receptor nuclear translocator (ARNT) (4 in Figure 1-10). This complex then binds to the xenobiotic response element (XRE) on the DNA (5 in Figure 1-10), altering expression of genes controlled by the XRE (6 in Figure 1-10) and initiating the transcription of genes for phase I (e.g. CYP1A) enzymes (7 in Figure 1-10) (Korashy and El-Kadi, 2006).

The transcription of phase II enzymes (e.g. glutathione transferase) is also initiated but to a much lesser extent than phase I enzymes. As a by-product of CYP1A enzyme activity, reactive oxygen species (ROS) are produced which cause damage to the cell and interrupt signaling pathways. Hydroxyl radicals can be formed through a Fenton reaction involving iron. These hydroxyl radicals can cause breaks in DNA strands, enzyme inactivation, and lipid peroxidation. However, there are mechanisms in place to regulate the activity of the AhR. One such mediator is a protein called the AhR repressor (AhRR), which is induced by the AhR and competes with it for ARNT and XRE, thus forming a negative feedback loop for the down regulation of AhR binding (Bernshausen et al., 2006).

TCDD is very persistent and once it is inside a cell, it can turn on the AhR signaling pathway and keep it turned on, resulting in the over-expression of CYP1A, and excess production of free radicals leading to cancer and teratogenicity in exposed organisms.
Figure 1-10: Receptor mediated metabolism of xenobiotic PAH or TCDD ligand by the aryl hydrocarbon receptor (AhR) in a liver cell. AIP, AhR inhibitory protein; HSP90, 90 kDa heat shock protein; ARNT, AhR nuclear translocator; XRE, xenobiotic response element; cyp1a/CYP1A, cytochrome P450 gene/protein (Scott, 2009).
Mixed function oxygenase (MFO) enzymes, measured as hepatic ethoxyresorufin O-deethylase (EROD) activity, is the function of CYP1A, a particular form of CYP enzymes that can be used as a biomarker for dioxin-like toxicity. In early life stages of fish, the visible signs of this toxicity are typically manifested as BSD. How these AhR pathways mediate such diverse biological responses through the same mechanism is not fully understood, but agonist potency, duration of pathway activation, life stage, and responsiveness of cells and tissues likely play a role (Elonen et al., 1998; Walisser et al., 2004).

1.4.2 CYP1A Independent Pathways

Activation of CYP1A enzymes is not the only mechanism of embryo toxicity. Other chemicals, such as the herbicide thiobencarb, cause similar cardiotoxic effects as CYP1A activity, but its metabolism is through a different set of enzymes, flavin monooxygenases (Arcand-Hoy and Benson, 1998). Pericardial edema and related vascular abnormalities do not seem to be exclusive to compounds eliciting CYP1A activity, and may not be mediated through the AhR (Villalobos et al., 2000). In addition, several biological or toxic effects, such as neurobehavioral problems, have been described for PCBs which are probably not related to an AhR mediated mechanism of action. These effects show distinctly different structure-activity relationships for PCBs than those observed for AhR binding involving multiple ortho-chlorine substitution or hydroxylated metabolites (Billiard et al., 2008).

The toxicity of complex mixtures of polycyclic aromatic hydrocarbons (PAHs) may occur through multiple mechanisms of action. Barron et al. (2004) found that the alkyl phenanthrene model better explained PAH toxicity in salmon and herring than either the narcosis or AhR models. Unlike dioxins, the developmental toxicity of PAH mixtures is not necessarily additive (Billiard et al., 2008). This is important for risk assessment because currently the approach for complex mixtures of PAHs usually assumes concentrations are additive (Billiard et al., 2008).

1.5 Blue Sac Disease (BSD)

BSD is a non contagious, chemically-induced syndrome first described in hatchery fish by Wolfe in 1957. It was originally thought of as an infectious disease (Atkinson, 1932), but Wolfe showed it to be
the result of exposure to ammonia. Researchers have shown BSD to result from toxicants other than ammonia, and it has been long-established as an effect of chemical exposure (Solomon and Weis, 1979; Wisk and Cooper, 1990).

The signs of BSD are similar among chlorinated aromatics, but the severity and particular order in which the signs of BSD present themselves may vary depending on the toxicant and the concentration. BSD causes an anaphylactoid response in hatched larvae due to increased permeability in the vascular endothelium that coincides with AhR-mediated CYP induction (Finn, 2007). Characteristic signs include: edema, hemorrhaging, tube heart, fin rot, and craniofacial and spinal deformities, which suggest membrane damage and circulatory failure. These malformations cause impaired development and ultimately result in mortality.

BSD in fish is similar to wasting syndrome in mammals and chick edema disease in birds. These diseases are the result of toxicity mediated by a common mode of action through the cytochrome P450 (fish and mammals) and P448 (birds) (CYP1A) systems. In all three classes of animals, toxicity is induced by exposure to dioxin and DLCs which activate ligand binding to the AhR (Gilbertson et al., 1991; Uno et al., 2004). The mechanism of gene activation, chronic activation of AhR, and subsequent toxicity are very similar in birds, mammals, and fish and typically result in reproductive failure and increased mortality. All vertebrates have a CYP 1A protein with similar numbers of receptors and binding affinity for TCDD, while invertebrates do not. Although there are differences in the signs expressed, the similarities indicate that BSD is common among all vertebrates. This common mode of toxicity will have implications to human health effects as humans also possess CYP1A enzymes and appear to be sensitive to dioxin toxicity (Birnbaum, 1994).

The signs and severity of BSD can also be an indicator of toxicity to a suite of TCDD-like contaminants including: polycyclic aromatic hydrocarbons and polychlorinated aromatic hydrocarbons (Walker and Peterson, 1991). Mehta et al. (2008) found that TCDD causes reduced blood flow in embryonic zebrafish due to blood regurgitation in the heart, suggesting some form of valve failure. After testing their theory they found that embryos exposed to TCDD failed to form valve leaflets as the heart
matured. In addition, Mehta and colleagues (2008) found that although TCDD did not block the formation of the bulbus arteriosus it prevented the normal growth and development of this important segment of the outflow tract. Exposure to TCDD also leads to altered localization of endothelial cells at the junctions of the atrioventricularus and the bulboventricularus. All of these effects may lead to the formation of a tube heart, hemorrhaging, the constriction of blood vessels, and the ultra filtration of blood leading to edemas.

These sub-lethal effects, some of which have been observed in in vitro fertilised eel embryos (Palstra et al., 2006), are poorly understood in eels and their impacts on wild populations are difficult to estimate. In natural environments, sub-lethal levels of contaminants can lead to critically impaired vision and swim bladder inflation, leading to the delayed recovery of eel populations. TCDD can be maternally transferred to the embryo during oogenesis, but alkyl PAHs are accumulated by direct contact with contaminated sediments or water (Brannon et al., 2006; Kukkinen et al., 2003).

The time of exposure during the development of an embryo seems to play a big part in the effects of AhR- agonist toxicity (McIntosh and Hodson, 2008; Scott and Hodson, 2008). If the exposure is post organogenesis, the embryo is more able to metabolize and eliminate the chemical and thus experiences a less severe response (McIntosh et al., 2008). Walker et al. (1994) found that lake trout embryos exposed to DLCs were usually able to develop normally for the first 8-10 weeks, but one week before hatch, eggs began to show signs of toxicity that escalated during the next two to three weeks (Cook et al., 1994). This response suggests that the AhR is functioning in very early life stages of fish, as indicated by the induction of cytochrome P4501A in the cardiovascular endothelium of lake trout embryos at least 1 week before hatch (Elonen et al., 1998). If the eggs make it to hatch, the fry usually die within a few weeks. Unfortunately, because there are “no dead bodies in the streets”, there are no alarm bells sounding to alert the public to the mass deaths of these embryonic fish.

1.6 Rationale and Study Objectives

The objectives of this study were to (1) assess whether chemicals accumulated by adult American eels growing to maturity in contaminated ecosystems have the potential to cause recruitment failure
through maternal transfer and toxicity to embryonic eels, and (2) to determine the spatial relationship between toxicity and American eels caught at five locations along the St. Lawrence River. In order to test these objectives, I needed to perform bioassays of toxicants on fish embryos. Because eels are not yet routinely bred in captivity and their eggs have not yet been collected in the wild, I was unable to use their embryos for direct analysis. Japanese medaka however, are a reliable test organism and easy to acquire and maintain in the lab. Medaka also have a transparent chorion which allows for non-invasive observations of development to be made. For these reasons the Japanese medaka was used as a surrogate species for the American eel.

The goals of this study were three-fold. First, I wanted to establish the sensitivity of medaka embryos to injected 2,3,7,8-TCDD. This would establish a standard dose-response curve with which to compare eel tissue extracts to. This bioassay would allow the concentration of dioxin equivalents in fish extracts to be estimated. Second, I wanted to determine if concentrations of DLCs in eel tissues were toxic enough to toxicity of eel tissue extracts increases along the St. Lawrence from east to west following a gradient of chemical contamination.

The main questions I hope to answer are; (1) are DLCs sufficiently concentrated in eels to be embryo-toxic to medaka? (2) Is toxicity to embryos unique to L. Ontario eels or should we expect to find similar results in other contaminated areas? (3) Will the dose toxic to fifty percent of exposed medaka (ED50) fall below the concentration extracted from our caught eels? If chemicals accumulated by adult eels growing in L. Ontario are transferred maternally to their eggs at concentrations sufficient to cause recruitment failure, then I can hypothesize that:

- Extracts of L. Ontario eels should be toxic to eggs of other species of fish (i.e. medaka).
- Toxicity should vary with the concentration of dioxin and DLCs in the extracts.
- Extract toxicity should increase with eel size, and therefore fatty tissue content.
- Extract toxicity should increase as eels age, spending more time exposed to contaminants.
Extract toxicity should be lower in eels that reside further away from L. Ontario, i.e., eels in the Margaree River would be least toxic. In Figure 1-11, I have developed a simple model to illustrate why I believe eel reproduction is affected by chemicals.
Figure 1-11: A working model of chemical effects on eel reproductive success. These factors most likely lead to recruitment failure of juvenile American eels to freshwater habitats.
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Chapter 2

Development of standard methods for assessing American eel (Anguilla rostrata) tissue extracts via nano-injection into Japanese medaka (Oryzias latipes) embryos

Abstract

Dioxin-like contaminants (DLCs) accumulate to high concentrations in the tissues of the American eel (Anguilla rostrata). The maternal transfer of these contaminants to offspring may exacerbate the already complex nature of their population decline. To model the toxicity of maternally-transferred chemicals in laboratory toxicity tests, I adapted an egg-injection technique with newly fertilized eggs of Japanese medaka (Oryzias latipes). I assessed several factors that may affect the outcome of tests, using TCDD as a standard test chemical. These included optimal methods for exposure, rearing, and signs of TCDD toxicity; time-course of exposure, injection volume tolerance, egg agitation; and TCDD toxicity tests. Heart rate measurements were also collected to determine whether heart rate was a good indicator of dioxin-like toxicity.

Our results suggest that (1) placing injected medaka eggs in scintillation vials on a rotary shaker was the optimal rearing method to obtain the most synchronized, successful and consistent hatch time; (2) injections of medaka eggs can take place up to seven hours post fertilization (between the 1 cell and late high blastula stage) without any treatment bias as long as the treatments are randomized; (3) injecting medaka eggs with large volumes (up to 10 nL) of extract did not affect estimates of TCDD toxicity; (4) medaka respond in a dose-dependent manner to injected doses of TCDD, and; (5) heart rate is not a reliable indicator of dioxin-like toxicity to medaka embryos.
2.1 Introduction

Recruitment of American eel, *Anguilla rostrata*, to L. Ontario has declined by 99% since the early 1980’s (Dekker, 2003). This resulted in the 2004 closure of the Ontario eel fishery and the listing of the American eel as ‘endangered’ under the Ontario Species at Risk Act in June 2008 (OMNR, 2007). One hypothesis to explain this population crash is embryo mortality due to maternally-derived contaminants, specifically 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and dioxin-like contaminants (DLCs) such as PCBs and chlorinated furans. Anguillid eels mature and develop for up to 40 years in freshwater habitats that are often contaminated by industrial pollutants; their high fat content enables the absorption and storage of large amounts of persistent hydrophobic contaminants. Eels are also benthic, predatory, and semelparous. These factors may all contribute to their sensitivity to lipophilic contaminants.

To test the embryo mortality hypothesis, a standard toxicity test is needed with a surrogate species since eel embryos are not readily available for testing. I chose Japanese medaka (*Oryzias latipes*) as a teleost model due to their low maintenance cost, year round egg production, and transparent chorion which facilitates detailed, non-invasive observations of development (Gonzalez-Doncel et al., 2004). Medaka are also particularly sensitive to xenobiotics such as dioxin congeners (Chen and Cooper, 1999), making them suitable indicators of developmental toxicity. However, standard methods for assessing toxicity, egg exposure, and post treatment rearing must first be determined.

This study utilized the nano-injection technique described in detail by Akerman and Balk (1995) and Walker et al. (1996). Most toxicity testing on medaka embryos involves topical or immersion application of chemical solutions (Helmstetter et al., 1995a; Helmstetter et al., 1995b; Elenon et al., 1998; Owens, 2000), but the actual dose transported into the developing embryo is difficult to estimate. These methods also do not represent maternal transfer of contaminants to embryos and there is a potential bias due to differential uptake of chemicals with different properties across the chorion. In contrast, injections accurately deliver a known dose directly into the embryo’s yolk sac shortly after fertilization, and they closely simulate the maternal transfer of persistent contaminants to offspring. However, in preliminary
testing, five variables were identified that might affect the outcome of the test. In the present study I developed and refined methods for nano-injection of eel tissue extracts into medaka eggs as a bioassay of embryotoxicity. The purpose of this study was to 1) refine the methods of the nano-injection technique so that they can be applied to eel extract injections, 2) calibrate the response of medaka to injected TCDD, and 3) assess whether heart rate was a relevant indicator of TCDD toxicity.

*Ideal post treatment incubation method*

The transparent chorion and embryo of medaka eggs allow for non-invasive observations to be made throughout all stages of embryogenesis. The eggs can tolerate a wide range of temperatures (7-38°C), allowing the researcher to control the rate of development (Albert, 1998). However, hatching time also varies considerably depending on light, dissolved oxygen concentrations, type of test container, and agitation (Downing and Litvak, 2002; Yang and Yang, 2004). To synchronize hatching among treatments without affecting the toxicity of DLCs, I compared time to hatch among different agitation regimes to identify the regime that provided consistent times to hatch.

*Injection time course*

The injection of multiple treatments into hundreds of eggs immediately after fertilization requires several hours, during which time the embryos begin to develop. For this reason, it was important to understand the effect of time from fertilization on toxicity of injected chemicals. In the present study, dioxin toxicity to medaka embryos was assessed by injections 1 to 7 hours post fertilization.

*Volume tolerance experiment*

Toxicity tests with medaka eggs typically involved injection of 1 nL of test substance into each egg of about 1000 nL volume (Walker *et al.*, 1996). To test the toxicity of maternally derived contaminants at one eel equivalent (1 eeq), the extracts must be concentrated 1000 fold and solvent exchanged to triolein, the carrier. However, it is very difficult to concentrate the extracts further to extend the bioassay to higher concentrations, specifically 10 eeq. Therefore I assessed whether injecting 10 nL, 10 times the typical volume, would cause adverse effects on medaka embryos or change the observed toxicity of TCDD.
**Dioxin standard curve**

The response of medaka embryos to DLCs extracted from eel tissue must be calibrated against 2,3,7,8-TCDD, the most potent congener of the DLCs. This allows an estimate of the relative toxicity of eel extracts in toxic equivalents (TEQs). The potency of DLCs is based on their toxicity relative to TCDD, which, as the most toxic congener, is given a value of 1 toxic equivalent factor (TEF) (Safe, 1990). To establish the sensitivity of medaka embryos as a bioassay organism, medaka eggs were injected with six concentrations of 2,3,7,8-TCDD from 0.625 to 15 pg TCDD/nL. This test was repeated four times.

**Heart rate as an indicator of toxicity**

The primary mechanism of dioxin-like toxicity is through binding of compounds with the aryl hydrocarbon receptor (AhR), also known as the dioxin receptor. AhR is a ligand activated factor which initiates transcription of an array of genes, including those for cytochrome P450 proteins and reactive oxygen. Signs of toxicity include; yolk sac edema, pericardial edema, tube heart, ocular edema, body hemorrhaging, fin rot, spinal deformities, reduced blood circulation, and craniofacial malformations, all of which are manifestations of a non-contagious syndrome known as blue sac disease (BSD). Cardiac tissue appears to be a primary target of dioxin-like toxicity, which suggests that heart rate may be a convenient indicator of the extent of dioxin toxicity (Antkiewicz et al., 2005). To determine whether heart rate was a sensitive indicator of dioxin-like toxicity, embryos were injected with a range of TCDD and the heart rates were counted daily.

The following null hypotheses were tested in this experiment: (1) shaking eggs post injection would not promote the most consistent responses to injected chemicals; (2) injecting embryos up to seven hours post fertilization would not affect the sensitivity of the egg to the contaminant and its survival; (3) medaka eggs would not withstand injection volumes greater than 0.1% of its volume; (4) there would not be a dose-dependent response of medaka eggs to injected TCDD; (5) heart rate would not be affected by TCDD.
2.2 Materials and Methods

2.2.1 Experimental Design

Five separate experiments were conducted:

1. Egg agitation
   To determine the ideal rearing method that would elicit the most synchronized hatch time and highest survival rate without affecting toxicity of post injected medaka eggs, treated embryos (Golden strain, 12 eggs/treatment) were incubated with three rearing methods; shake, aerate and stationary.

2. Time injection post fertilization
   To establish if injection time had an effect on toxicity, separate groups of medaka embryos (12 eggs/ treatment) were injected 1, 3, 5 and 7 hours after fertilization with 7.5 pg TCDD/nL and a triolein control.

3. Volume tolerance
   To determine if the volume of triolein injected was harmful to eggs (12 eggs/treatment) at fixed doses of TCDD (0 and 15 pg/nL), medaka eggs were injected with increasing volumes of triolein (1, 2, 8, 10, 24 nL) containing decreasing concentrations of TCDD. The dose was delivered with a single penetration of the egg followed by multiple depressions of the foot pedal to deliver the appropriate amount.

4. TCDD calibration curve
   To calibrate the medaka injection test, and develop a dose-response curve for TCDD with which to compare injected eel extracts, newly fertilized medaka embryos (18 egg/dose, 1 nL injection volume) were chronically exposed to a range (0.625-15pg/nL) of TCDD concentrations.

5. Heart rate as an indicator of toxicity
   To determine whether heart rate (b/m) is an accurate measure of dioxin-like toxicity, newly fertilized medaka embryos were exposed to a range of TCDD concentrations (0.625-15pg/nL) and heartbeats were counted daily across all treatments and compared to other indicators of toxicity.
2.2.2 Chemicals

A stock solution of 2,3,7,8-tetrachlorodibenzo-p-dioxin (99% pure, 50 µg/ml solution in toluene) was purchased from AccuStandard, Inc. (New Haven, CT). A dosing solution was prepared by solvent exchange of the toluene-TCDD stock solution into dichloromethane (DCM) and then sterile triolein (injectable oil) followed by serial dilution into six dosing concentrations of TCDD (15, 7.5, 3.75, 1.875, 1.25, and 0.625 pg/nL) and a DCM/triolein control (detailed in Appendix A). The TCDD concentration of a sample of 7.5 pg/nL was confirmed to be accurate ± 1 pg/nL (n= 3) by J. Byer, Department of Chemistry, Queen’s University, using Gas Chromatography- isotope dilution- high resolution Mass Spectrometry (GCMS). The concentrations were selected based on the practical dilution of a limited volume of chemical on the assumption that the medaka specific ED50 for TCDD was 1.572 pg/nL based on a waterborne exposure (Elonen et al., 1998). Triolein, 1m2m3-Tri[cis-9-octadecenoyl]glycerol (>99% pure), was purchased from Sigma (St. Louis, MO) and was selected because it caused minimal needle clogging and carrier-related mortalities while providing a good solvent for lipophilic contaminants (Walker et al., 1996).

2.2.3 Needle Preparation

Quartz glass needles with filaments (QF 100-70-10, Sutter Instrument Co., Novato, CA) were prepared at the United States Geological Services laboratory in Columbia, MO. At Queen’s University, the needles were beveled at a 20° angle on a micro pipette beveller (BV-10 Sutter Instrument Co., Navato, CA). Beveled needles were coated with Sigmacote® (a silicon solution in heptanes, Sigma-Aldrich St. Louis, MO), to reduce clogging by egg yolk. Injection needles were back-loaded with 8 µL of dosing solutions as close to injection time as possible using an Eppendorf microloader (detailed in Appendix A).

2.2.4 Medaka Egg Production

Adult medaka (Golden strain from Carolina Biological Supply Company (Burlington, NC)), were maintained in dechlorinated water at 26 ± 1°C in a re-circulated system with a photoperiod of 16 h light: 8
Fish were fed live brine shrimp (*Artemia salina*) (Ocean Star International, Inc.) three times a day and Nutrafin Basix staple flake food™ 1 hour after the onset of the photoperiod to induce oogenesis. Eggs were collected as soon as possible after fertilization by gently stripping them from the females with the dip net still submerged in water. Medaka eggs were pooled from as many as 70 different females, from 15 aquaria containing four separate populations of the same strain of medaka (Golden strain). These populations consisted of fish from Arofish Supply Co., Hamption, NH; Trent University, Peterborough, ON; and Queen’s University Kingston, ON, fish from each source were kept isolated to maintain the genetic integrity of the population. Inbreeding was minimized by allowing fish from different tanks, but from the same source, to mix every few generations. Eggs were placed in embryo rearing solution (ERS) and examined microscopically to determine if they were viable. Between breeding, the fish were separated into male and female tanks, the water temperature was dropped to 25 ± 1°C to simulate non-breeding season, and the fish were switched to flake food.

### 2.2.5 General Bioassay Procedures

The typical bioassay method is described below. Certain aspects of these methods were modified and expanded to refine the bioassay to the particular needs of fish tissue extract injections. These methods were used throughout the following five experiments unless otherwise noted.

#### 2.2.5.1 Egg Preparation and Injection

Eggs were injected according to Åkerman & Balk (1995) and Walker et al. (1996). Newly fertilized eggs were immobilized in holes punched in a layer of agar. Eggs were covered with ERS, a combination of salts and methylene blue mold inhibitor (Carolina Biological Supply Co., Burlington, NC) (Appendix B), for 10-20 minutes. Viable eggs (did not stain blue) were injected individually 2-4 hours after fertilization with the triolein/TCDD solutions using a micromanipulator (MX110R, Siskiyou Corporation, Grants Pass, OR) at a 60° angle under a stereomicroscope and a Pico-Injector (PLI-100, Harvard apparatus St. Laurent, QC).
2.2.5.2 Needle Calibration

Each injection needle was calibrated by dispensing a small volume of the triolein/TCDD solution into ultra-pure water and measuring the diameter of the spherical droplet using the microscope’s eyepiece micrometer. The injection volume was estimated by assuming a perfect sphere \((\pi d^3/6)\). Assuming the egg volume was 1023 nL, 0.1 % of the egg volume would be 1.0 nL, equivalent to 112.5 µm at 4.5 times magnification. By adjusting the injection duration and pressure, the dosing volume could be accurately delivered every time. The balance pressure of the Pico-Injector was maintained greater than the internal pressure of the egg to prevent vitelline fluid from entering the injection needle upon insertion into the egg and to provide accurate and consistent volume delivery of the dosing solution.

2.2.5.3 Egg Rearing

The injected eggs were transferred to 20 mL borosilicate glass scintillation vials (Fisher scientific) with 15 mL of clean ERS (dissolved oxygen content of 8.7 mg/L, conductivity of 180 µs/cm and pH of 7.76). The vials were capped with Teflon-lined lids and placed on a Mistral Multi-mixer (Lab-line instruments, Inc.) set to roughly 90 rpm and incubated at a room temperature of 26 ± 1°C with a photoperiod of 16 L: 8 D and 24-h renewal of ERS. The duration of the bioassay was set at 11 days post fertilization (dpf) to ensure that most embryos had hatched by this time but had not begun to starve; the day the eggs were fertilized and injected was considered day 0. Every day after injection the embryos were observed for mortalities, signs of toxicity and hatchlings. On the eleventh day of the bioassay the embryos were scored for signs and severity of BSD and then sacrificed by an overdose of tricaine methanesulfonate (MS222, Sigma), an anesthetic.

2.2.5.4 Scoring Eggs For Toxicity

On day 11 of each experiment, eggs were inspected under a Leica MZ95 (Meyer Instruments, Houston TX) compound microscope and images were taken using SPOT advanced imaging software (Ver. 4.7, Diagnostic instruments Inc. Sterling Heights, MI). Double blind scoring was performed to reduce bias. Treatment vials were covered with tin foil and randomly assigned a letter from A to G. The
vials were then shuffled and randomly selected for scoring (not scored in order from A-G). Eggs were scored for signs and severity of BSD modified for medaka from Khan (2007) and detailed in Appendix A. Signs of toxicity included: pericardial, yolk sac, and ocular edema; body and yolk hemorrhaging; fin rot; craniofacial and, spinal deformities; tube heart; and reduced blood flow. These signs were given a score between 0-3 (3 being the worst case), 0-2 (2 being the worst case) or 0-1 (presence or absence). Yolk sac (YE) and pericardial edema (PE) were scored on a 0-3 scale because the embryos showed a broad range of responses allowing a discrimination of mild and severe responses. Tube heart (TH) and blood circulation (CIRC) were scored from 0-2 because there were only two variations of these signs from normal. Ocular edema (OE), spinal malformations (S), craniofacial malformations (CF) and fin rot (FR) were all scored on a scale of 0-1 (presence or absence) due to the difficulty of judging a gradation in their severity (Figure 2-1 b and c). The maximum possible score for each fish was 15, the sum for all signs of pathology. Premature hatching (Figure 2-1 d) induced by TCDD was observed in this study as well as by Chen and Cooper (1999) but was not included in the BSD scoring because it was accounted for by the other features of the index. Mortalities that occurred on day 0 and 1 were considered non-treatment mortalities and attributed to injection trauma because the heart had not yet begun to beat, so that toxicity was not the likely cause of death.

2.2.5.5 Calculating BSD Score and Index

Individual fish scores for signs of pathology were averaged to give treatment BSD scores for live fish. Mortalities after day 1 were assumed to be associated with BSD because in TCDD-exposed fish, they followed the onset of signs of BSD. Dead fish were assigned the maximum score for BSD (15) plus 0.5; therefore the highest possible BSD score when mortality was considered was 15.5. The BSD index is the BSD score, including mortalities, normalized to the highest possible score (15.5) (Appendix A). This creates a measure of the overall response on a 0-to-1 scale (modified from Scott et al., 2008), and avoids the bias created by basing BSD scores on a diminishing number of surviving fish at higher doses.
**Experiment 1: Rearing of embryos post injection**

Medaka eggs (n= 12) were exposed to four treatments: injection of 1 µL of triolein, puncture (sham injected), and a 1 hour waterborne exposure to 3.42 ng TCDD/µL followed by a control. The eggs were placed in one of three rearing conditions; stationary Petri dish, shaker, or aeration, at 25 ± 1°C in ERS with a photoperiod of 16 h light: 8 h of dark. For the waterborne exposure, 36 eggs were placed in a test tube with 10 mL of 3.42 ng/mL of 2,3,7,8-TCDD in water for one hour. The eggs were blotted on a Kim wipe to remove excess TCDD and placed in clean ERS. Eggs were immersed in waterborne TCDD for the positive control because injection needles were not available at the time. Twelve eggs were placed in each of the three rearing methods. For the shaker treatment, rearing followed the aforementioned general bioassay protocol. For the aeration method, 12 eggs were placed in 40 mL ERS in a 50 mL plastic test tube with a Pasteur pipette connected to an air hose inserted to the bottom. The air flow was roughly 128 bubbles/minute. For the stationary method, 12 eggs were placed in 20 mL ERS in a covered Petri dish and placed on a shelf. Incubation followed the general bioassay protocol for all treatments. This entire experiment was repeated three times.

**Experiment 2: Injection time course**

Injections of 0 pg TCDD/nL and 7.5 pg TCDD/nL were performed between 1 and 7 hours post-fertilization (hpf) at two-hour time intervals (1, 3, 5, 7 hpf). The 7.5 pg TCDD/nL exposure was used to determine a difference in sensitivity to dioxin, depending on what molecular and cellular events were affected by dioxin. The triolein control was used to observe the effect of time without toxicity being a factor. To inject 12 eggs takes about 5 minutes. Twelve eggs were injected at the beginning of the hour with triolein and another 12 eggs were injected with dioxin immediately after. These experiments were repeated three times and it was assumed that there was no interaction between injection stress and toxicity.
Figure 2-1: Images of medaka embryos taken 11 dpf and after TCDD injection. Scale bars represent 1 mm. (a) Normal embryo, straight spine, upturned jaw and absorbed yolk sac. (b) Embryo exposed to 7.5 pg/nL TCDD exhibiting 1) string heart, 2) yolk sac edema, 3) fin rot 4) craniofacial malformations. The arrow indicates jaw malformation and blunt nose. (c) Embryo exposed to 15 pg/nL TCDD with many of the same signs of toxicity as (b); 1) yolk sack edema, 2) ocular edema, and 3) pericardial edema. (d) Prematurely hatched embryo with ruptured yolk sac and severe spinal and craniofacial deformities. Premature hatching was observed in both the 7.5 and 15 pg/nL treatments.
**Experiment 3: Injection volume tolerance**

Standard procedures for egg injections state that the target injection volume is typically 0.1% of the egg’s internal volume to avoid physical stress to the egg (Walker et al., 1996). Japanese medaka eggs measured 1.2 mm in diameter, weighed 1.1 mg, and had a calculated volume of 1.023 µL, thus 0.1% of that egg volume was 1.0 nL. Sterile triolein (2, 8, 10, 20, 30, 40, 100 nL) was injected in 7 groups of medaka (n =12) to determine if injection volume affected embryo survival and expression of toxicity, and to determine the greatest injection volume causing no effect with respect to the control (1 nL). Five concentrations of TCDD equivalent to 15 pg/nL were injected at 1, 2, 4, 8, and 24 nL to determine if injection volume interacted with toxicity expressed as blue sac disease (Table 2-1). This experiment was repeated three times.

**Table 2-1. TCDD concentrations to deliver a constant dose at ranging injection volumes.**

<table>
<thead>
<tr>
<th>Concentration (pg/nL)</th>
<th>No. of injections (nL)</th>
<th>Total dose (pg/egg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>7.5</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>3.75</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>1.875</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>0.625</td>
<td>24</td>
<td>15</td>
</tr>
</tbody>
</table>

**Experiment 4 and 5: TCDD bioassay and heart rate observations**

Medaka eggs were exposed to six concentrations of TCDD (0.625, 1.25, 1.875, 3.75, 7.5, 15 pg TCDD/nL) by nano-injection. Negative controls included a puncture of the chorion and vitelline membrane with the injection needle (sham injection) as well as a triolein/DCM control (0 pg TCDD/nL). Heart rates were counted daily for three randomly selected embryos in each treatment. Larvae were allowed to “rest” for about one minute before heart rates were measured. A hand-held tally counter and
stopwatch were used to record heartbeats for 15 seconds. Data were expressed as heartbeats per minute and pooled to obtain treatment means. This experiment was repeated four times.

2.3 Statistical Analyses

Statistical analyses including t-tests, sigmoidal concentration-response curves, linear regression, ANOVAs, and ED50s were calculated using Graphpad Prism software (Ver. 4.02, GraphPad Software, Inc., San Diego, CA, USA). LC50s were established using Probit Analysis (LC50 BAS 2.0, 1986, US EPA, Washington, DC). An alpha level of 0.05 was used for all statistical tests. To reduce pseudo-replication, eggs were collected from females in different tanks located in various locations around the room and on different days. True replication may not have been achieved because there were insufficient new solutions and new needles for each replicate.

2.4 Results

Experiment 1: Ideal incubation method post treatment

TCDD caused a severe reduction in survival of medaka eggs relative to control fish who survived on average 87 ± 18 % percent of the time (average n= 30). An analysis of variance showed that rearing method had no significant effect on survival (p = 0.76) except for TCDD-exposed eggs incubated in the still Petri dish method (p < 0.001) (Figure 2-2). These eggs had 0% survival by day 11 in the still method as opposed to 12% and 15% survival for shake and aerate respectively (Table 2-2). This suggests that stationary incubation may affect the toxicity of TCDD in medaka eggs. Rearing method had a significant effect on time to hatch (p= 0.01). Embryos incubated in the shaker had the most synchronized hatch time (11.1 ± 0.75 days, average n= 30). While the aeration method also produced early and somewhat synchronized hatch times (11 ± 2.3 days, average n= 30), there was more variability with regards to survival and the apparatus itself was cumbersome and difficult to standardize. Time to hatch was extended without shaking or aeration.
Figure 2-2: Affect of incubation method on the time to hatch of medaka eggs post exposure with error bars (average n= 30). * Indicates a significant difference compared to ‘shake’ and ‘aerate’ in hatch time. Embryos reared in the still method hatched significantly later than embryos reared in either the shake or aeration methods. Embryos exposed to TCDD and ‘not agitated’ all died.

Table 2-2. Percent survival of embryos exposed to four treatments; injection, puncture, and waterborne TCDD followed by a control, and reared in three incubation methods; shake, aerate, and still with 95% confidence intervals (average n= 30).

<table>
<thead>
<tr>
<th>Treatment/incubation method</th>
<th>Shake</th>
<th>Aerate</th>
<th>Still (Petri dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inject triolein</td>
<td>76 ± 21</td>
<td>65 ± 17</td>
<td>92.7 ± 12</td>
</tr>
<tr>
<td>Puncture</td>
<td>83.3 ± 18</td>
<td>91.8 ± 0.2</td>
<td>81.2 ± 15</td>
</tr>
<tr>
<td>TCDD</td>
<td>12*</td>
<td>15 ± 8*</td>
<td>0.00*</td>
</tr>
<tr>
<td>Control</td>
<td>72.7 ± 30</td>
<td>84.3 ± 22</td>
<td>87.3 ± 18</td>
</tr>
</tbody>
</table>

* Indicate significant reductions in percent survival.
Experiment 2: Injection time course

Our results supported the hypothesis that injecting eggs up to seven hours post fertilization would not significantly affect survival or signs of toxicity. Linear regressions showed no significant difference with injection time after fertilization in hatch success \((p = 0.81)\) in either the 0 or 7.5 pg TCDD/nL treatments (Figure 2-3). Similar trends were seen with signs of toxicity, percent normal and mortality \((p = 0.17, 0.12, \text{ and } 0.19, \text{ respectively; data not shown})\). This allowed injections to take place up to seven hours post fertilization with no significant effect between treatments as long as the treatments were randomized. The DCM/triolein control injection (0 pg TCDD/nL) confirmed the assumption that there was no interaction between injection stress and toxicity.

Experiment 3: Volume tolerance

A second order polynomial regression showed there was no significant difference in mortality \((p = 0.25)\) or BSD index \((p = 0.7)\) between injections of 1 nL and 10 nL of triolein, suggesting that a 1% injection volume would not affect toxicity or survival. An analysis of variance showed that increasing injection volumes of various doses of TCDD from 1 nL to 8 nL were not significantly different \((p = 0.12)\) in signs of toxicity (circled points on Figure 2-4) despite a 27% increase in sensitivity. There was also no significant difference in mortality \((p = 0.13, \text{ data not shown})\). The response at 10 nL of TCDD was predicted to be 0.67 using the second order polynomial non-linear equation of the line: \(Y = 0.63 + (0.83 \times \log_{10} (\text{volume})) - (0.79 \times \log_{10} (\text{volume})^2)\). This was very similar to the BSD index of 0.63 when 1 nL of 15 pg TCDD/nL was injected. These responses imply that 10 nL of 1 eeq is equivalent to 10 eeq. The response at 10 nL needed to be estimated because 1.5 pg TCDD/nL was not available. A significant reduction \((p = 0.03)\) in BSD occurred with injections of 24 nL of 0.625 pg TCDD/ nL, suggesting that volume interacts with toxicity when injections exceed 10 nL (Figure 2-4). This may also mean that volumes greater than 10 nL were far less accurate at delivering the appropriate dose. Similar patterns were seen with percent mortality and percent normal embryos (data not shown).
Figure 2-3: Cumulative hatch for TCDD-injected and control embryos when there was a 1 to 7 hour delay after fertilization in injecting the eggs. Error bars represent standard deviation (average n= 30).

Figure 2-4: Effect of injection volume on signs of toxicity (BSD), with error bars expressed as a second order polynomial non-linear regression (average n= 30). The numbers next to each TCDD point indicate the concentration injected in pg TCDD/nL; the total dose of TCDD was constant (15 pg/mg egg). The circled points represent the non-significant values of injection volumes. The dashed line represents the predicted response to a 10 nL injection of a theoretical 1.5 pg TCDD/nL dose. The equation of the polynomial regression for TCDD is; \( Y = 0.63 + (0.83 \log_{10} (\text{volume}) - (0.79 \times \log_{10} (\text{volume})^2) \). The equation of the polynomial regression for triolein is; \( Y = 0.13 + (0.001 \log_{10} (\text{volume})) + 0.001 \times \log_{10} (\text{volume})^2 \).
Experiment 4 and 5: TCDD bioassay and Heart rate as an indicator of toxicity

As TCDD concentration increased, so did the presence and severity of BSD and heart rate. Signs of BSD were clearly evident in the 7.5 and 15 pg TCDD/nL treatments with severe pericardial edema. The first signs of toxicity occurred on day four for both 7.5 and 15 pg TCDD/nL treatments and included delayed development, tissue opacity (associated with necrosis), blood pooling in the caudal fin, and granular globules floating free between the yolk and chorion. This timing corresponded with the development of the liver rudiment (Chen and Cooper, 1999), suggesting that the AhR is present and activated at this early stage of development. By day five, some embryos began to show chorion edema, defined as fluid surrounding the embryo and separating it from the chorion. This often results in the embryo being reduced in size. By day six, yolk sac edema, pericardial edema, tube heart, and blood regurgitation at the heart could be seen; regurgitation causes reduced circulation. Signs of toxicity were more severe by days 7-9, with reduced hatching and craniofacial malformations occurring by day 10. Craniofacial malformations appear to be one of the most sensitive signs of toxicity, occurring in treatments as low as 0.625 pg TCDD/nL. Most signs continued to worsen until day eleven when the embryos were scored for the final time. By this time embryos also exhibited spinal deformities, fin rot, and ocular edema.

Embryos treated with > 3.75 pg TCDD/nL had greatly reduced development and displayed abnormal hatching. They often hatched prematurely (day 5 or 6), late (day 12), or not at all, and frequency of abnormal hatching also increased in these embryos. Normal hatching is defined as tail first, but TCDD-exposed fish often hatched head first, yolk sac first, or did not complete hatch. Some animals died half-hatched and were classified as non-hatch. Early mortalities due to injection trauma (mortalities before day 2) were on average 13 ± 3% across treatments (n= 445), but were not significantly different from 0 (linear regression of mortality against dose \( p = 0.17 \)). The 11 day ED50 with 95% C.I. for BSD severity was 3.79 (1.34-6.39) pg TCDD/mg (Figure 2-5); the 11 day LD50 was 9.57 (6.71 - 13.65) pg TCDD/mg. The confidence intervals were derived by multiple linear regressions. When the ED50s were calculated rep by rep, the mean ± 95% limits of four ED50s was 4.18 ± 0.9 ng/mg (Appendix C), reflecting differences
among the reps in the distribution of data points at each dose, but overall a high degree of repeatability. The ED50 based on the average response at each dose (3.79 ng/mg) provided the clearest dose-response relationship.

The percentage of embryos showing normal development had a strong negative correlation with TCDD dose (Figure 2-6). Normal embryos were defined as hatchlings or eggs that were alive and showed no signs of BSD at day 11. This was not without bias though, as one must assume an egg is “normal” despite the inability to see all signs of toxicity. This assumption was supported by preliminary findings that compared scores of embryos as eggs on day 11 and scores for the same embryos as hatchlings on day 13. There was no significant difference between the two scores ($p=0.48$, $n=22$). There was no significant difference in heart rates among any of the TCDD treatments ($p = 0.24$). Heart rates of treated embryos increased over time in a predictable and linear manner, but when compared to the control, there was no significant difference ($p=0.39$) (Figure 2-7). Irregular heartbeats (non-rhythmic) however, were observed in embryos treated with greater than 1.875 pg TCDD/mg.

To assess the quality of the standard curve, all nine signs of BSD were analyzed for their frequency and correlation to toxicity (Appendix C). It was evident that while several signs increased in a dose dependent manner (yolk sac edema, pericardial edema, body hemorrhaging, circulation, and tube heart) others did not (ocular edema, spinal deformities, fin rot and craniofacial malformations). This may indicate that certain signs are more suitable indicators of toxicity and in future bioassays, focus should be placed on identifying their presence and severity.

Injection mortalities

Average injection mortalities and the estimated Y-intercept for time trial, volume tolerance, and TCDD bioassay experiments are shown in table 2-3. Linear regressions showed that injection mortalities were not affected by injection time, TCDD injection volume or TCDD concentration ($p = 0.5$, 0.6, and, 0.1 respectively) (data not shown).
Figure 2-5: The 11 day ED50 of the average BSD index for four repetitions of TCDD-injected embryos was 3.79 pg/mg with 95% C.I. of 1.3-6.4 with error bars (average n= 48). The Equation of the sigmoidal dose-response curve is:

$$y = \frac{1}{\frac{1}{10^{(0.5789-x)}} \times 1.879}$$  where x = logarithm of concentration and y= response.

Figure 2-6: Percent normal for TCDD injected medaka with error bars (average n= 48). The 11 day ED50 of the average percent normal for four reps was 1.75 pg/mg with 95% C.I. of 0.65-5. The Equation of the sigmoidal dose-response curve is:

$$y = \frac{100.1}{1+10^{(0.24-x)}}$$  where x = logarithm of concentration and y= response.
Figure 2-7: Heart rate (b/m), with error bars (n=18), of TCDD (pg/nL) injected embryos as a percentage of the control over 11 days. Heartbeats began two days post fertilization. The slopes of regressions relating heart rate to days post fertilization were not significantly different between TCDD treatments and control (p=0.39).

Table 2-3. Average injection mortality for the four experiments requiring injections.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Mean %</th>
<th>Y-intercept (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48</td>
<td>3 ± 4</td>
<td></td>
</tr>
<tr>
<td>Time trial</td>
<td>36</td>
<td>11 ± 4</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>Volume tolerance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triolein</td>
<td>36</td>
<td>28 ± 7*</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>Volume tolerance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCDD</td>
<td>36</td>
<td>10 ± 1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>TCDD bioassay</td>
<td>72</td>
<td>13 ± 3</td>
<td>10 ± 2</td>
</tr>
</tbody>
</table>

Control indicates background embryo mortality on days 0 and 1 of un-injected eggs.

* Indicates a significant increase in injection mortalities with increasing volume.
However, there was a significant ($p=0.02$) increase in injection mortality with increasing injection volume of triolein (2-40 nL). Because the slope for triolein injection volume was significantly different from zero, it is likely that early mortalities were due to injection stress, and probably a function of egg quality. An average injection mortality of $13 \pm 3\%$ is typical of injections assays. Tillitt et al. (2003) had similar injection mortalities of about 10% for control injections and slightly higher mortalities of 13% for extract injections.

2.5 Discussion

The results of this study have allowed us to determine: (a) the ideal incubation method for injected eggs; (b) the optimum injection period for fertilized eggs; (c) the tolerance of medaka eggs to increasing injection volumes; (e) the toxic potency of injected 2,3,7,8-TCDD, (f) as well as the sequence of lesions caused by TCDD; and (g) whether heart rate is an accurate measure of toxicity.

Incubation methods

It was believed that the shaker method for incubating post-exposed eggs would produce the greatest survival and earliest hatch time without affecting toxicity. Our results support this hypothesis. The observed early and synchronized hatching of eggs reared by the aeration and shaking method could be explained by mechanical agitation, water circulation and increased oxygen content. Mechanical agitation of the embryo’s hatching glands causes the release of chorionase, an enzyme which causes the chorion to break down in preparation for hatching. The hatching glands are located inside the mouth, pharyngeal cavity, and on the inner surface of the operculum of the embryo, and develop around 3-4 dpf (Leung and Bulkly, 1979). Shortly before hatch, forceful opercular movements begin, causing the disintegration of gland cells and the release of chorionase (Ishida, 1944). During the current experiment, the constant mechanical agitation of the eggs by shaking and rolling may have stimulated the hatching glands in the same way, causing the embryos to hatch in synchrony. Chorionase acts to break down the egg shell from the outside as well as the inside, causing a positive feedback-loop so that the more eggs
that hatch the more chorionase will be released, promoting additional egg hatching (Farwell et al., 2006).

Both the shake and aeration methods promoted water circulation within the test vials, presumably causing more eggs to come in contact with chorionase and synchronizing hatch.

Rolling of the eggs due to aeration can also cause early hatching due to the increased oxygen content of the rearing medium which increases the rate of yolk utilization in the embryo (Gonzalez-Doncel et al., 2004). The same can be said for the shaking method, as shaking a vial will also increase the oxygen content of the rearing medium.

While both the aeration and shaker methods produced low mortality and synchronized hatching around day 11, the aeration method was much more cumbersome. It was difficult to maintain consistent air flow to all of the treatments and the carrying capacity for the aeration method was only 10 vials. The stationary method elicited high survival but produced the slowest hatch times (on average over 15 days) and may have affected the toxicity of TCDD to exposed eggs as seen in the 0% survival of TCDD exposed eggs. Considering all factors, the shaker method was preferred for rearing medaka eggs post TCDD exposure due to its simplicity, carrying capacity (a single shaker can hold 100 vials), and consistency with respect to hatch time and survival rate. An early hatch time allows more experiments to be run. Repeating the experiment with injections of 15 pg TCDD/nL rather than using waterborne exposure, would demonstrate whether exposure method affects the response to aeration or shaking.

Injection time course

It was believed that injecting eggs up to seven hours post fertilization would not significantly affect survival or signs of toxicity. This hypothesis was supported by our results which demonstrated no systematic change in TCDD toxicity or the onset of hatching among groups of eggs injected at increasing intervals of time after hatch, up to seven hours post fertilization. Therefore, injections of newly fertilized medaka embryos may take place throughout the course of day zero as long as the treatments to be injected are randomized as a precaution against undetected bias of a time effect.

Volume tolerance
It was predicted that injecting 1% of the egg volume (10 nL) would not affect the survival of eggs. It was also believed that there would be no significant difference in BSD index between injecting 1 nL of 15 pg TCDD/nL and 8 nL of 1.88 pg TCDD/nL. These hypotheses were supported by our results. At a constant dose of TCDD (15 pg/mg), increasing volumes of carrier solvent (triolein) had no effect on toxicity up to 8 nL/egg, but a volume of 24 nL/egg reduced toxicity. Toxicity appears to be a function of dose per gram of lipid when greater than 8 nL of injection solution was delivered to an embryo. At volumes greater than 8 nL/egg, the triolein may have reduced the overall toxicity of the dose by acting as a reservoir of TCDD. An injection of 24 nL of 0.625 pg TCDD/nL almost elicited the same response as a control dose of 24 nL of triolein (Figure 2-4). Thus, the dosage accuracy significantly decreases with increasing volume and therefore smaller volumes and smaller number of injections are more precise. Injection volumes ≤ 8 nL were not large enough to affect toxicity or survival of embryos, indicating that injecting up to 10 nL (1%) of the egg volume would probably be a suitable procedure to achieve higher doses of TCDD or extracts of eel tissues. These data demonstrate classic toxicity principles; the response is stable up to a threshold, after which the response changes. The second order polynomial regression equation used for both the TCDD and triolein data may not be the best model to apply to these data. A ‘hockey stick’ model may accurately define the threshold, but in this case, there are inadequate numbers of doses for the analysis (only 5).

To ensure an accurate and consistent injection volume among all treatments, the injection dose was calibrated before each treatment using the microscope eyepiece micrometer. A precise droplet size could be produced that was 1 ± 0.01 nL (or 112.5 ± 2.25 µm). To further ensure volume injection accuracy, the droplet injected into each egg was measured after each injection. If it was not 112.5 ± 2.25 µm, the egg was discarded and the needle was recalibrated. The injection volume only changed if the needle became clogged with egg yolk or was broken (but still usable).

**TCDD Bioassay**

I predicted that there would be a dose dependent increase in toxicity and that heart rate would be affected by TCDD and thus a reliable and sensitive indicator of dioxin-like toxicity. The results supported
the former hypothesis but not the heart rate hypothesis. Medaka embryos were particularly sensitive to TCDD, doses as low as 1.88 pg/mg caused occasional lesions. The 11-day ED50 to cause BSD and the 11-day LD50 of TCDD with 95% C.I. were 3.79 (1.34-6.39) and 9.57 (6.71 - 13.65) pg TCDD/mg respectively. Similar ED50 values were observed for TCDD exposed medaka in other studies (Table 2-4). It should be noted that Tillitt et al., (2003) measured an ED50 value of 2.72 pg/mg based on injection. This is the most comparable to the ED50 value of 3.79 pg/mg in the present study and falls within our confidence intervals.

Table 2-4. Summary of ED50 values for medaka embryos exposed just after fertilization to waterborne or injected 2,3,7,8-TCDD. For waterborne exposure, doses were estimated from radioisotope labeled TCDD.

<table>
<thead>
<tr>
<th>Exposure method</th>
<th>ED50 (pg/mg of egg wt)</th>
<th>End point</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injected</td>
<td>3.79 (1.3-6.4)</td>
<td>BSD index</td>
<td>This study</td>
</tr>
<tr>
<td>Waterborne</td>
<td>0.24</td>
<td>Lesions</td>
<td>Wisk &amp; Cooper, 1990</td>
</tr>
<tr>
<td>Waterborne</td>
<td>1.2</td>
<td>Lesions</td>
<td>Elonen et al., 1998</td>
</tr>
<tr>
<td>Waterborne</td>
<td>1.9</td>
<td>Inhibition of swim bladder inflation</td>
<td>Kim &amp; Cooper, 1999</td>
</tr>
<tr>
<td>Injected</td>
<td>2.72</td>
<td>100 h post hatch</td>
<td>Tillitt et al., 2003</td>
</tr>
</tbody>
</table>

TCDD caused hemorrhaging, edemas, and body malformations in embryonic medaka, signs that were observed in a similar study by Wisk and Cooper (1990). The early signs of toxicity usually started around day five, coinciding with the development of the liver rudiment and included delayed development, tissue opacity, blood pooling, and granular globules in the yolk. These signs progressed until the endpoint of the experiment on day eleven, when the signs of toxicity included pericardial, yolk sac and ocular edema, tube heart, fin rot, spinal deformities, and craniofacial malformations.

Non treatment mortalities (excluding injection trauma) were observed in control treatments (2 ± 0.6%; n= 1697), which may have been due to a change in environmental conditions within the exposure vessels, toxicity (contaminated oil, needles, or a mix-up in solutions), disease, fungus, mishandling of eggs (e.g. sharp physical jolts), or starvation of hatched fry.
The frequency of occurrence of the nine signs of toxicity was assessed and it was evident that while five signs increased in a dose dependent manner (yolk sac edema, pericardial edema, body hemorrhaging, circulation, and tube heart), four of the signs did not (ocular edema, spinal deformities, fin rot, and craniofacial malformations). The five signs with a high degree of correlation may in fact lead to lethality, particularly circulatory failure and pericardial edema. The remaining four signs may result from interference in development (i.e., secondary to circulatory failure) or to experimental bias. Embryos injected with 7.5 and 15 pg TCDD/nL had significantly reduced ($p=0.03$, $p<0.01$ respectively) hatch success at 11 dpf, and scoring un-hatched embryos was more difficult than scoring hatchlings, especially for ocular edema (OE), spinal deformities (SD), fin rot (FR) and cranial facial malformations (CF). This could explain the drop in BSD score of these signs at 7.5 and 15 pg/nL. The onset of mortality occurred at day 5 for the 15 pg TCDD/nL- treated embryos, followed closely on day 6 by the 7.5 pg/nL treatment. Mortality however, was much less severe for the 7.5 pg/nL treatment (Figure 2-8). The majority of mortalities for all treatments occurred between day 6 and day 8 when the yolk sac was being absorbed and the injected oil droplet was beginning to be metabolized. Mortalities within the control group were occasionally seen late in the experiment and could not be attributed to any known factor.

Reduced hatch rate and incomplete hatch were observed in the higher treatments. This may be explained by the inhibition of hatching gland cell differentiation or gland cell necrosis (Ishida, 1944). In this study, jaw deformities and ocular edema were frequently observed in embryos exposed to $\geq 1.875$ pg TCDD/mg. If these deformities interfered with gland cell production or function, this could potentially reduce hatch success. Reduced and incompletely-hatched embryos have also been observed in other fish species exposed to TCDD (Walker and Peterson, 1991). Increased exposure to injected TCDD caused an increase in pericardial edema which typically led to an increased severity of tube heart. This is supported by Spitsbergen et al. (1991) who identified the cardiovascular system as the initial tissue affected by TCDD in lake trout embryos. Scott et al. (2009) and Cantrell et al. (1996) also report that the embryonic vasculature is a physiological target for TCDD- induced DNA damage and apoptotic cell death in medaka.
Figure 2-8: Average percent cumulative mortality of TCDD exposed embryos over the 11 day study period (average n= 48). Mortalities in treatments 7.5 and 15 pg/nL began early on and greatly increased with time.
Although the heart was severely affected by TCDD exposure, the heart rate did not differ among treatments. These results do not support the hypothesis that heart rate would be a reliable and sensitive measure of TCDD toxicity. While this may seem counter-intuitive, this was also observed by Scott et al. (2008) and Hornung et al. (1999), the latter of whom exposed rainbow trout sac fry to TCDD. Heart rate is therefore not an indicator of TCDD toxicity.

There was a dose-dependent increase in toxic effects that was not observed until the liver rudiment was formed in the embryos at day four. This stage-dependent toxicity was consistent with TCDD induction of the AhR in liver cells leading to CYP1A enzyme production (Wisk and Cooper, 1990; Chen and Cooper, 1999). These results supported our earlier prediction that medaka embryos would respond in a dose-dependent manner to injected TCDD. The background activity of BSD in embryos varied in each test. These values ranged from 0.00 to 0.25 but were not significantly different among replicates \((p = 0.11)\). Injection mortalities, although not significantly different among treatments, were fairly high (9-17%) compared to previous nano-injection studies which reported ≤ 1% mortality (Walker et al., 1996). This could be due to methodological discrepancies between the studies, differences in size and species of eggs, our relative inexperience with the injection technique, dull injection needles, or the handling of the eggs post injection. To reduce mortalities in future injection studies, the individual performing the injections should practice injecting and beveling needles for at least one month prior to an experiment.

A sample size of 18 rather than 12 eggs was selected for the bioassay and future eel extract treatments to compensate for relatively high injection mortalities and occasional (2% of the time) non-treatment mortalities. Also, 18 eggs is conveniently half of an agar tray.

The measured toxicity of TCDD to medaka embryos was within the range of reported data for medaka (Table 2-4) and other fish species (Table 2-5). Based on their LD50’s, medaka are less sensitive to TCDD exposure than rainbow trout, lake trout, fathead minnow, and largemouth bass. This rank order of species sensitivity is confirmed by Elonen et al. (1998). One discrepancy can be seen in Olivieri and Cooper’s (1997) findings of fathead minnow with an LD50 of 25.71 pg/mg, more than twice as high as
the current medaka LD50 of 9.57 pg/mg. Based on the ranking by Elonen et al. (1998) and the LD50 of the current study, Olivieri and Cooper’s (1997) value would appear to be too high.

Validation of egg injection

The primary route of accumulation of lipophilic, environmental contaminants in fish eggs is from the maternal transfer of these contaminants during oocyte maturation (Miller, 1993). Whether injection of newly fertilized eggs with lipophilic contaminants is comparable to this natural exposure route is an important question. Research by Walker et al. (1994); Akerman and Balk (1995); and Wilson and Tillitt (1996) support nano-injection of lipophilic contaminants as an accurate measure of the maternal transfer of these toxicants to eggs during oocyte maturation. This strengthens the validity of using egg injection data in environmental risk assessment and its use in estimating embryotoxicity of American eel tissue extracts. These results were also supported by Edmunds et al. (2000) who suggested that direct nano-injection into the yolk sac of newly fertilized oocytes provides an in-vivo approach that closely parallels the maternal transfer of lipophilic contaminants and exposes embryos to contaminants during a sensitive developmental period.

Table 2-5. Summary of LD50 and ED50 values for TCDD exposed embryos of various fish species. Exposure was via waterborne TCDD or by injection (indicated below). For waterborne exposures, doses were estimated from radioisotope labeled TCDD.

<table>
<thead>
<tr>
<th>End point</th>
<th>Fish species</th>
<th>Dose (pg/mg) / effect</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD50- injected</td>
<td>Medaka</td>
<td>9.57</td>
<td>This study</td>
</tr>
<tr>
<td>ED50- injected</td>
<td>Medaka</td>
<td>3.79</td>
<td>This study</td>
</tr>
<tr>
<td>LD50- injected</td>
<td>Rainbow trout</td>
<td>0.23- 0.49, depending on strain</td>
<td>Walker &amp; Peterson, 1991</td>
</tr>
<tr>
<td>LD50-injected</td>
<td>Rainbow trout</td>
<td>0.171-0.374 depending on strain</td>
<td>Zabel et al., 1995</td>
</tr>
<tr>
<td>ED50- waterborne</td>
<td>Fathead minnow</td>
<td>0.14, 7-day ED50 for minor and severe lesions</td>
<td>Olivieri &amp; Cooper, 1997</td>
</tr>
<tr>
<td>LD50-waterborne</td>
<td>Fathead minnow</td>
<td>25.71*</td>
<td>Olivieri &amp; Cooper, 1997</td>
</tr>
<tr>
<td>ED50- injected</td>
<td>Lake trout</td>
<td>0.065, endothelial CYP1A induction in sac fry</td>
<td>Guiney et al., 1996</td>
</tr>
<tr>
<td>LD50- injected</td>
<td>Lake trout</td>
<td>0.047 (for hatchlings)</td>
<td>Guiney et al., 1996</td>
</tr>
<tr>
<td>LD50- injected</td>
<td>Largemouth bass</td>
<td>4.57</td>
<td>Tillitt et al., 2003</td>
</tr>
<tr>
<td>LD50- injected</td>
<td>Mummichog</td>
<td>0.25</td>
<td>Toomey et al., 2001</td>
</tr>
</tbody>
</table>

*Indicates an unusual LD50 value for Fathead minnow.
Conclusions

The results indicate that a maternal transfer as low as 3.79 pg TCDD/mg may have sublethal effects on medaka embryos. This baseline relationship can be used for future comparisons of a variety of unknown dioxin-like toxicants from environmental samples, such as eel extracts and complex mixtures, allowing for TEQ estimation. Although the cardiovascular system of medaka is clearly affected by TCDD, heart rate was not, and is therefore not a reliable measure of toxicity. These results appear to be reliable due to the controls and internal standards that were performed, as well as the accuracy and precision of dosing. Based on the results of the five experiments in the current study, it is recommended that the ideal method for assessing eel tissue extracts via nano-injection into medaka eggs are as follows:

- Medaka embryos should be injected prior to seven hours post fertilization to reduce treatment bias and treatments should be randomized
- Injection volumes should be no greater than 10 nL (or 1% of the egg volume), but a typical injection volume is 1 nL
- Once injected, the medaka eggs should be carefully transferred into scintillation vials, capped and reared on a shaker for 11 days (if eggs are fertilized the morning of day 0)
- Post injected embryos should be checked daily for mortalities, signs of toxicity and hatchlings.
- 50% of the ERS should be changed daily
- On day 11 post injection, the fish should be scored for signs of toxicity whether they have hatched or not
- The signs and severity of toxicity that should be scored are (in order of importance): pericardial, and yolk sac edema; tube heart, circulation, hemorrhaging, spinal and craniofacial deformities, fin rot, ocular edema, and percent normal
- The resulting BSD index should be compared to the standard TCDD index for medaka, so that the relative concentration of DLCs in eel tissues can be estimated
2.6 References


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Chapter 3 Determining the spatial trend in contaminants accumulated by American eels (*Anguilla rostrata*) and if they are embryotoxic to Japanese medaka (*Oryzias latipes*)

Abstract

Using the early life stages of Japanese medaka (*Oryzias latipes*) as a surrogate species, I compared the toxicity of American eel (*Anguilla rostrata*) tissue extracts to that of 2,3,7,8-TCDD. Sexually maturing, large eels were sampled in 2007 and 2008 from five locations along the St. Lawrence River and Lake Ontario as well as the Hudson River, United States, and Canal Dessel-Schoten, Belgium. By injecting eel extracts into newly fertilized medaka eggs, I assessed developmental problems associated with the maternal transfer of toxicants to offspring. Medaka eggs were injected immediately after fertilization with 1 or 10 nL of eel extract and on 11 days post injection, and scored for signs of toxicity. The objectives were to (1) determine if eel tissue extracts were embryotoxic to medaka and (2) determine if there was a spatial gradient in toxicity along the St. Lawrence River. Eel extracts from all collection sites caused no dioxin-like toxicity in Japanese medaka embryos. There were however, significant mortalities and reduced hatching at higher doses from some eels. This may indicate that while dioxin-like contamination may no longer be a problem, other contaminants may still affect embryo survival. The reduction in maternal tissue contamination indicated by this bioassay is mirrored in chemical monitoring of POPS in L. Ontario fish. It would imply that recruitment of juvenile eels into L. Ontario should be increasing; this can be seen in the growing number of eels climbing the Moses Saunders eel ladders at Cornwall, Ontario from 2003 to the present day.
3.1 Introduction

The decline of the American eel (Anguilla rostrata) fishery in L. Ontario in 2004 prompted its listing as endangered under the Ontario Species at Risk Act in June 2008 (OMNR, 2007). Due to their complex life cycle, little is known about eels once they leave their freshwater habitats and migrate to the Sargasso Sea to mate. However, it is certain that all American eels as well as their European (Anguilla anguilla) and Japanese (Anguilla japonica) cousins are experiencing severe population declines. Among the many theories to explain this decline, chemical contamination is the primary focus of the present research. Scientists have long been aware of this global decline (Castonguay et al., 1994; Castelnaud et al., 1994), but much work is still needed to understand the causes and to apply appropriate management plans for eel recovery. Eels inhabit many political jurisdictions as well as international waters, making management strategies difficult to synchronize. For example, Ontario has closed the L. Ontario fishery due to a lack of eel abundance but the coastal areas of New Brunswick and Quebec still have open, active harvests.

Recently, spawner quality has been proposed to play a key role in the decline of the species. Pollution by chemical substances such as dioxin-like contaminants (DLCs) (polychlorinated dioxins, and furans as well as PCBs) may have a large impact on the reproductive success of the eel (Couillard et al., 1997; Maes et al., 2005). Once in the water, these persistent, lipophilic chemicals can adsorb to particulate organic matter, precipitate to sediments, and slowly accumulate up the food chain. The concentration of contaminants biomagnifies at each trophic level, causing toxicity at the highest levels of the food web. In the past (1940’s-1980’s), L. Ontario sediments and biota were heavily contaminated with DLCs (Cook et al., 2003). Lake trout were particularly affected; the entire population was extirpated from L. Ontario in the 1950s due to lamprey predation, and lack of recovery following lamprey control has been attributed to embryotoxicity of DLCs (Cook et al., 2003). Because of the similarities between lake trout and eels, it is possible that DLCs are also responsible for the decline in eel recruitment.

The accumulation of lipophilic contaminants while eels are developing in their freshwater habitats may be the primary cause for the reduced quality and fitness of spawners entering the breeding
grounds (Geeraerts and Belpaire, 2009). While this theory is well understood and is receiving a great deal of attention in Europe, its application to the American eel and the maternal transfer of contaminants in eels is not well understood. Most of the current information on American eels has been extrapolated from lake trout and the European eel. There are two schools of thought on the decline of the Atlantic eel. Europeans are concerned with whether the parents successfully migrate to the breeding grounds, while North Americans are focused on whether the offspring are successfully recruiting to continental waters.

Our study objective was twofold, (1) to determine if dioxin and DLCs are in high enough concentrations in sexually maturing, large eels to be embryotoxic to their offspring; and (2) to determine if there is a spatial gradient of extract toxicity along the St. Lawrence River (SLR) and if L. Ontario eels are the most contaminated.

To address these objectives, whole adult eels from seven locations were homogenized and lipophilic contaminants extracted from their tissues. This tissue extract was solvent exchanged into triolein (non-toxic, injectable oil) and diluted serially into four doses injected into newly fertilized medaka embryos to simulate maternal transfer. The embryos were scored 11 days after injection for signs and severity of toxicity including; edema, hemorrhaging, craniofacial and spinal deformities and reduced blood flow. All of which are characteristic signs of dioxin-like toxicity, are non-reversible, and cumulatively are referred to as blue sac disease (BSD). The score of BSD and BSD plus mortality (BSD index) were the measurements of toxicity in the current study.

This approach implied two assumptions: first, that eel sensitivity to dioxin-like embryotoxicity was greater than that of medaka, an assumption that risks a false negative. Recent data in our lab supports this assumption based on a similar response of a contaminant induced biomarker (EROD) in eels to rainbow trout (Cutler, 2009) which are three times more sensitive than medaka (Elonen et al., 1998). Second, I assessed that the dose administered was equivalent to the dose in tissue, i.e., there was no loss during injection or excretion by the egg. This assumption was supported by visual observation of the egg immediately after injection and was confirmed by confocal imaging with spectrofluorometry of an injected fluorescent label (Appendix C).
fluorescent label into various tissues and organs of the embryos (pigment cells, gallbladder, and yolk), identifying which areas were likely to be contaminated with eel extract injections.

This study will help to determine whether tissue extracts from large resident yellow and silver eel emigrants cause teratogenic effects on fish early life stages, and what contaminants may be causing toxicity. The results will be important to management plans for restoring the American eel population and for effective remediation of contaminated sites.

3.2 Materials and Methods

3.2.1 Experimental Design

To assess whether American eels are a source of lipophilic contamination to their offspring, Japanese medaka (Golden strain), embryos were exposed to graded doses of eel tissue extracts immediately after fertilization using nano-injection exposure (18 eggs/dose, 1 and 10 nL injection volumes). I assumed that contaminants were transferred with 100% efficiency from eel tissue to triolein vehicle, i.e., that there was no loss of contaminants during the lipid extraction, solvent exchange or serial dilution processes.

3.2.2 Location of Sample Sites

This experiment was designed for the spatial comparison of eel contamination along the SLR and tributaries to the Gulf of St. Lawrence (Figure 3-1). Sample sites were selected based on either known chemical contamination of the ecosystems or assumed levels of contamination based on watershed characteristics or the behavior of eels (Table 3-1). The European eels (Anguilla anguilla) collected from Canal Dessel-Schoten, Belgium, by Claude Belpaire, and the U.S. American eel collected from the Hudson R. by the US Fish and Wildlife Service (organized by Amy Roe, USFWS, Cortland NY) were selected as a positive control due to their presumed high level of contamination. The characteristics, including total toxic equivalence (TEQ) to 2,3,7,8-TCDD, for the eels sampled from each location are listed in Table 3-2.
Figure 3-1: Map showing eel collection sites. The Hudson River, NY site is not shown on this map. Black stars indicate very contaminated sites, dark grey stars indicate contaminated sites and circled light grey stars indicate reference sites. The abbreviations indicate the following water bodies; LO- Lake Ontario, RO- Riviere Ouelle, RSO- Riviere Sud Ouest, NB- New Brunswick (Miramichi River), NS- Nova Scotia (Margaree River), HR- Hudson River, Be- Belgium (Canal Dessel-Shoten).
Table 3-1. Contaminated and uncontaminated sample sites with a brief explanation of their contaminant history.

<table>
<thead>
<tr>
<th>Site</th>
<th>Location</th>
<th>Contaminated/Reference site</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. Ontario</td>
<td>Ontario</td>
<td><strong>Contaminated</strong>- Mirex from Hooker Chemical Co., Niagara falls, NY, TCDD/Fs from Dow Chemical, Midland, MI, and Aleris Specification Alloy Products, Mississauga, ON, pesticides from agriculture, PCBs from wastewater treatment facilities, retene, furans and chlorinated hydrocarbons from pulp and paper production, atmospheric deposition (1,2,3,4,5).</td>
</tr>
<tr>
<td>R. Ouelle</td>
<td>Quebec</td>
<td><strong>Contaminated</strong>- Eels caught in the St. Lawrence estuary tidal zone weirs are silver eels migrating from L. Ontario and the watershed of the St. Lawrence River. Their tissue extracts are dominated with chemical profiles typical of L. Ontario, and their origin is confirmed by the presence of Mirex, unique to L. Ontario.</td>
</tr>
<tr>
<td>R. Sud-Ouest</td>
<td>Quebec</td>
<td><strong>Reference</strong>- Watershed flows through forested and agricultural region, this was assumed to have low inputs of PCBs (6).</td>
</tr>
<tr>
<td>Miramichi R.</td>
<td>New Brunswick</td>
<td><strong>Contaminated</strong>- Creosote, PAHs, and PCBs from wood treatment and power generation as well as Domtar pulp and paper production, heavy metals from Heath steel base-metal mine, inadequate wastewater treatment, DDT from forest protection, and industrial history (7, 8).</td>
</tr>
<tr>
<td>Margaree R.</td>
<td>Nova Scotia</td>
<td><strong>Reference</strong> – Watershed flows through forested and agricultural region. Little information is given for contaminants in this river, but abundant salmon stocks (9) indicate a relatively uncontaminated environment.</td>
</tr>
<tr>
<td>Hudson R.</td>
<td>U.S.A</td>
<td><strong>Heavily contaminated</strong> with PCBs from General Electric at Albany, NY; heavy metals, furans, dioxin, pesticides, and PAHs from sewage discharges and urban runoff (10, 11).</td>
</tr>
<tr>
<td>Canal Dessel-Schoten</td>
<td>Belgium</td>
<td><strong>Contaminated</strong> with organic pesticides, PCBs, industrial organic compounds, and heavy metals; sources include chemical industries (12).</td>
</tr>
</tbody>
</table>

Table 3-2. Characteristics of eels sampled from seven collection sites (Byer et al., 2009).

<table>
<thead>
<tr>
<th>Site</th>
<th>N</th>
<th>Collection year</th>
<th>Avg. length (cm)</th>
<th>Avg. weight (g)</th>
<th>Tot. TEQs (pg/g ww)</th>
<th>Avg. % lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. Ontario</td>
<td>5</td>
<td>2008</td>
<td>118 ± 5</td>
<td>2829 ± 444</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>R. Ouelle</td>
<td>5</td>
<td>2007</td>
<td>115 ± 6</td>
<td>3046 ± 375</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>R. Sud-Ouest</td>
<td>4</td>
<td>2008</td>
<td>92 ± 17</td>
<td>1518 ± 1019</td>
<td>0.7</td>
<td>19</td>
</tr>
<tr>
<td>Miramichi R</td>
<td>5</td>
<td>2007</td>
<td>75 ± 6</td>
<td>845 ± 206</td>
<td>0.9</td>
<td>17</td>
</tr>
<tr>
<td>Margaree R.</td>
<td>5</td>
<td>2007</td>
<td>69 ± 6</td>
<td>657 ± 162</td>
<td>0.8</td>
<td>18</td>
</tr>
<tr>
<td>Hudson R.</td>
<td>5</td>
<td>2008</td>
<td>62 ± 3</td>
<td>474 ± 64</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Belgium</td>
<td>5</td>
<td>2008</td>
<td>80 ± 4</td>
<td>1033 ± 113</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA: not available

Preparation of Tissue Extracts

Five of ten eel extracts were selected based on the largest and presumably oldest and most contaminated fish from each location. Whole eels were homogenized and their lipophilic contaminants were extracted into dichloromethane (DCM) by soxhlet. Lipids were removed by gel permeation chromatography (GPC) and silica gel columns (Appendix D). The extracts were tested for seven dioxins, ten furans, and twelve PCBs (Table 3-3).

Eel tissue is remarkably high in lipid content (20-30% by weight), making solvent extraction and sample clean-up very time consuming. Two laboratories were involved in the chemical extraction; The Analytical Services Unit (ASU), Queen’s University, and The National Water Research Institute, Environment Canada, Burlington Ontario. To ensure that the contaminant extraction methods were similar between the two laboratories; the toxicity of eel tissue extracts prepared by both laboratories were compared. One eel collected from Riviere Ouelle (RO-3) was homogenized; half of the homogenate was prepared by Environment Canada while the other half was prepared by the ASU. These extracts were injected into medaka embryos and scored for BSD. There was no significant difference ($p = 0.61$) between BSD indices for extracts prepared by either laboratory, indicating that samples prepared at both laboratories were comparable.
Table 3-3. List of lipophilic, DCM extractable contaminants measured for each eel.

<table>
<thead>
<tr>
<th>Dioxins</th>
<th>Furans</th>
<th>PCBs (congener No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2378 TCDD</td>
<td>2378 TCDF</td>
<td>77</td>
</tr>
<tr>
<td>12378 PeCDD</td>
<td>12378 PeCDF</td>
<td>81</td>
</tr>
<tr>
<td>123478 HxCDD</td>
<td>23478 PeCDF</td>
<td>126</td>
</tr>
<tr>
<td>123678 HxCDD</td>
<td>123478 HxCDF</td>
<td>169</td>
</tr>
<tr>
<td>123789 HxCDD</td>
<td>123678 HxCDF</td>
<td>105</td>
</tr>
<tr>
<td>1234678 HpCDD</td>
<td>234678 HxCDF</td>
<td>114</td>
</tr>
<tr>
<td>OCDD</td>
<td>123789 HxCDF</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>1234678 HpCDF</td>
<td>123</td>
</tr>
<tr>
<td>OCDF</td>
<td>1234789 HxCDF</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>OCDF</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td></td>
<td>167</td>
</tr>
<tr>
<td></td>
<td></td>
<td>189</td>
</tr>
</tbody>
</table>

Abbreviations: TCDD, Tetrachlorodibenzo-p-dioxin; PeCDD, Pentachlorodibenzo-p-dioxin; HxCDD, Hexachlorodibenzo-p-dioxin; HpCDD, Heptachlorodibenzo-p-dioxin; OCDD, Octachlorodibenzo-p-dioxin. Abbreviations with an –F ending are furans.

Each medaka egg weighed, on average 1 mg, so 1 eel equivalent (eeq) represented the extract from 1 mg of eel tissue/egg, injected in 1 nL; in other words the extract was concentrated 1000-fold. Tissue extract equivalent to 20 ± 1 g was solvent-exchanged into 20 µL of injectable triolein followed by serial dilution to concentrations of 1, 0.5, 0.25, and 0.12 eeq. These concentrations were chosen based on practical dilution factors of limited extract volume. To ensure the solvent exchange of eel extract into triolein was accurate, a sample of 1 eeq (1 µL) of RSO-5, RO-2, RO-3, LO-4, and LO-9 were sent to Environment Canada for analysis. There was no significant difference (p = 0.50) between total TEQs of the eel tissue homogenate before and after extraction and solvent exchange. These results support the assumption of 100 % efficiency in solvent exchange of eel contaminants into triolein.

To ensure that the toxicity test could detect small responses around 1 eeq, a dose of 10 eeq was administered by injecting 10 nL of 1 eeq. Previous experiments demonstrated that increasing the dioxin dose by 10-fold in 10 nL of triolein, increased toxicity by 10-fold, while 10 nL of triolein control caused
no effects (Chapter Two). This 10-fold volume increase was necessary because of the difficulty in concentrating the eel extract a further 10-fold due to limited eel tissue availability.

All stock solutions were made to 20 µL to facilitate accurate needle filling. Two needles per concentration were filled with 3.5 µL each using an Eppendorf microloader pipette, the second was a spare in case of breakage or clogging. Any remaining solution was rinsed out of the Eppendorf microloader pipette tip with DCM into the remaining solution in the conical bottom vial; the head space was filled with nitrogen and the vial was stored at -20 °C. The total volume of solution required per dose was 18 nL (1 nL in each of 18 eggs) plus another 10 nL for needle calibration, leaving 3.47 µL remaining in the needle. This solution was saved for analysis to verify accuracy of stock preparation.

3.2.3 Medaka breeding and egg collection

Medaka were maintained and eggs were collected as described in Chapter Two. Medaka eggs were pooled from as many as 70 different females, from 15 aquaria containing three separate populations of the same strain of medaka (Golden strain). Medaka were maintained in dechlorinated water at 26 ± 1°C in a re-circulating aquatic system with a photoperiod of 16 h light, 8 h dark. During experimentation, eggs were gently stripped from the females within 3 hours of “day break”. Viable eggs were placed one each in 36 holes punched out of agar in a gridded Petri dish. Eggs were covered with embryo rearing solution (ERS; Appendix B) to prepare them for injection. The salts in the ERS induce a slightly hypertonic environment in the egg, allowing the injection needle to enter the egg with minimal uptake of egg fluids.

3.2.4 Egg exposure

Eggs were exposed to extracts and reared following the methods outlined in Chapter Two, the eggs were injected with five concentrations of eel extracts (eeq) plus a triolein negative control and 15 pg TCDD/nL positive control. The volume of dosing solution delivered to each egg was 1 nL or 0.1% of the egg volume for the 0.125 eeq - 1 eeq treatments. The 10 eeq treatment however, received 10 nL or 1% egg volume, of the 1 eeq dose. Eggs were reared according to the methods in Chapter Two. Briefly, injected eggs were transferred into 20 mL vials containing fresh ERS, capped and placed on a shaker set
to the lowest speed (about 90 rpm). Eggs were maintained at 26 °C ± 1°C, with a photoperiod of 16:8 hours of light: dark. Vials were capped to reduce fungal infections.

3.2.5 Scoring eggs for toxicity

Scoring procedures for eggs injected with eel extracts were identical to those of eggs injected with TCDD, described previously in Chapter Two. Fry were scored for nine signs of toxicity indicative of blue sac disease; yolk sac, pericardial and ocular edema, body hemorrhaging, tube heart, craniofacial and spinal malformations, fin rot, and reduced blood circulation. These variables were assigned a score from 0-1, 0-2, or 0-3 based on the severity of the occurrence. Mortality due to BSD was given the highest score of 15.5. The scores were added up using the BSD index equation detailed in appendix C and normalized to the highest score to give a value from zero to one. Because ‘normal’ fish included eggs and hatchlings, the summation of percent hatch, normal, and mortality will exceed 100%.

3.2.6 Statistical Analysis

Signs of toxicity were analyzed for ED50s using GraphPad Prism software (Ver. 4.02, GraphPad Software, Inc., San Diego, CA, USA). The same program was used to perform statistical analyses including: t-tests (paired two sample for means), sigmoidal concentration-response curves, linear regression, and ANOVAs. Pseudo-replication was reduced using the same methods as in Chapter Two.

3.3 Results

Early mortalities due to injection trauma were on average 18 ± 2% (n = 3492, data not shown) in treatments injected with 1 nL; the slope of the relationship between dose and mortality was not significantly different from zero (linear regression \( p = 0.11 \)). Treatments injected with 10 nL had an average injection mortality of 22.5 ± 7% (n= 612) which was not significantly different (\( p = 0.35 \)) than the average injection mortality for 1 nL injections. Signs of dioxin-like toxicity, measured as BSD score, were not expressed by medaka embryos exposed to any of the eel extracts; all eel extracts scored lower than 3 out of 15.5. The percentage of normal embryos that were exposed to 10 eeq did not differ significantly among sample sites; however, there was a significant decrease in the percentage of embryos that were normal compared to the triolein control (\( p < 0.01 \)) (Figure 3-2).
Signs of non dioxin-like toxicity included: cyclopia and reduced eyes, triclops, elongated jaw deformities (instead of reduced jaw), inability to eliminate methylene blue (component of ERS) from urinary bladder or yolk sac, inability to inflate swim bladder, post hatch lethargy, and two-headedness (siamese twins). Some of these signs were present in many embryos (reduced eyes, blue urinary bladder, uninflated swimbladder, and lethargy) while others were only observed once or twice. Twinning (cephalodidymus), cyclopia, and reduced development of eye cup were observed in medaka embryos topically exposed to mixtures of pentachlorophenol and tributyltin chloride (Helmstetter et al., 1996). It is unlikely that these responses were due to errors in the solvent exchange method because signs of toxicity were present in the low treatments. If DCM was left behind during the solvent exchange method, there would be a dose dependent response in toxicity, which was not the case. These signs could possibly be random, or caused by other contaminants present in the extract such as polybrominated diphenylethers, fluorinated compounds, and other DCM-extractable contaminants.

Although the eel extracts did not cause dioxin-like toxicity in the medaka embryo bioassay, significant mortalities \( p = 0.002 \) were still observed in higher treatments compared to controls (Figure 3-3). Percent hatch did not differ significantly \( p= 0.76 \) among sites. However, there was a significant decrease in hatch success of the 10 eeq \( p=0.003 \) and 1 eeq \( p= 0.02 \) treatments compared to the controls (Figure 3-4). These results are summarized in Table 3-4.

Variability in the response of medaka embryos to injected eel extracts could be seen in their unusually low response (mortality) to relatively high TEQs (Figure 3-5), particularly RO-2, LO-2, and LO-4. By removing these unusual points, the linear regression becomes tighter with an \( R^2 \) value of 0.221 compared to 0.006, indicating there is a rough relationship between TEQs and mortality. Similar results were found with TEQ vs. BSD score and percent mortality (data not shown), suggesting that LO2, LO4 and RO2 extracts should be repeated.
Figure 3-2: Percent normal embryos with error bars (average n= 64) for individual eels from all seven sample sites exposed to 10 eeq. The * indicates a significant difference between percent normal at 10 eeq and the triolein control, considering all treatments combined. There is no significant difference among sites or between triolein and 10 eeq within sites. Abbreviations: BE, Belgium; HR, Hudson R.; LO, L. Ontario; RO, R. Ouelle; NB, New Brunswick; NS, Nova Scotia; RSO, R. Sud-Ouest; eeq, eel equivalent.

Figure 3-3: Percent mortality with error bars (average n= 64) of 10 eeq treatments from seven locations compared to their triolein controls. Treatments with the same letter are not significantly different. There is no significant difference among sites or between triolein and 1 eeq within sites. Abbreviations: BE, Belgium; HR, Hudson R.; LO, L. Ontario; RO, R. Ouelle; NB, New Brunswick; NS, Nova Scotia; RSO, R. Sud-Ouest; eeq, eel equivalent.
Figure 3-4: Percent hatch with error bars (average n= 64) for medaka embryos exposed to 1 and 10 eeqs. Treatments with the same letter are not significantly different. There is a significant difference between the triolein control and both the 1 and 10 eeq treatments, considering all treatments combined.

Abbreviations: BE, Belgium; HR, Hudson R.; LO, L. Ontario; RO, R. Ouelle; NB, New Brunswick; NS, Nova Scotia; RSO, R. Sud-Ouest; eeq, eel equivalent.

Table 3-4. Summary of results including average percent mortality, normal embryos and BSD score (/15.5) for medaka eggs exposed to 10 eeq (mean ± 95% CI).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>% Mortality</th>
<th>BSD score</th>
<th>% Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triolein control</td>
<td>20</td>
<td>7.84 ± 2.1</td>
<td>0.22 ± 0.2</td>
<td>84.7 ± 6.4</td>
</tr>
<tr>
<td>15 pg TCDD/nL control</td>
<td>20</td>
<td>84.23 ± 8.6</td>
<td>7.10 ± 2.2</td>
<td>2.25 ± 1.6</td>
</tr>
<tr>
<td>BE</td>
<td>5</td>
<td>39.44 ± 25.6</td>
<td>0.28 ± 0.4</td>
<td>55.36 ± 23.2</td>
</tr>
<tr>
<td>HR</td>
<td>5</td>
<td>27.62 ± 25</td>
<td>0.83 ± 0.7</td>
<td>45.88 ± 26.1</td>
</tr>
<tr>
<td>LO</td>
<td>5</td>
<td>20.09 ± 23.7</td>
<td>0.49 ± 0.7</td>
<td>60.82 ± 30.9</td>
</tr>
<tr>
<td>RO</td>
<td>5</td>
<td>25.81 ± 17.8</td>
<td>0.16 ± 0.2</td>
<td>57.77 ± 21.5</td>
</tr>
<tr>
<td>NB</td>
<td>5</td>
<td>15.02 ± 14.5</td>
<td>0.03 ± 0.05</td>
<td>65.28 ± 31.1</td>
</tr>
<tr>
<td>NS</td>
<td>5</td>
<td>16.31 ± 9</td>
<td>0.40 ± 0.3</td>
<td>55.12 ± 6.9</td>
</tr>
<tr>
<td>RSO</td>
<td>5</td>
<td>35 ± 33.9</td>
<td>0.56 ± 0.6</td>
<td>51.56 ± 27.3</td>
</tr>
</tbody>
</table>

Abbreviations: BE, Belgium; HR, Hudson R.; LO, L. Ontario; RO, R. Ouelle; NB, New Brunswick; NS, Nova Scotia; RSO, R. Sud-Ouest.
**Figure 3-5:** Percent mortality of medaka embryos exposed to 10 eeq against total TEQ values for each eel (average n= 16). TEQ values were calculated using TEFs for each congener detected (Byer, 2009). Abbreviations: LO, L. Ontario; RO, R. Ouelle; NB, New Brunswick. Hudson R., and Belgium data were not included in this figure because TEQs were not available for these eels.
Total TEQs ranged from 1.54-6.50 pg TEQs /g ww (mean = 3.50 pg TEQs/g ww) (Byer, 2009).

Exponential regressions of TEQs against individual fish size, showed a significant correlation between weight (p <0.001; Figure 3-6) and length (p <0.001; data not shown), supporting the assumption that larger fish are more contaminated then smaller fish. Figure 3-6 shows that eels from specific sites are grouped together, with the exception of RSO-5. The tight grouping of L. Ontario and R. Ouelle eels supports our assumption that R. Ouelle eels are most likely from L. Ontario. Similar groupings were also found with regressions of percent mortality at 1 and 10 eeq against weight however, there were no significant correlations.

3.2.3 Quality Assurance

Unexpected mortalities at lower concentrations prompted a repeat of 12 unusual data points (Appendix E) that resulted in lower mortalities for 11 of the 12 points confirming a negative response. As in Chapter Two, the injection volume was consistent, based on constant measuring of the dose droplet during injection. This was evident from the precise TCDD values in Table 3-4, suggesting reliable methods and an accurate estimation of the response. The eel extract values for percent mortality, BSD score and percent normal in Table 3-4 were also very consistent, indicating reliable data and bioassays. Triolein controls had much lower mortality, BSD score, and higher percent normal than most treatments. This may not be due to a chemical or dose related affect, but there is still something distinctly different between eel extract and triolein exposed fish. Among the eel extracts there appears to be a weak, yet not significant location specific response.
Figure 3-6: Exponential regression of TEQ against weight of sexually maturing American eels sampled from five locations along the St. Lawrence Seaway (Byer, 2009). The abbreviations indicate the following water bodies; NS- Nova Scotia (Margaree R.), NB- New Brubnswick (Miramichi R.), RSO- R. Sud-Ouest, LO- L. Ontario, RO- R. Ouelle. The equation of the line is: $Y = 0.58e^{(0.0006 - X)}$.
3.3 Discussion

There did not appear to be sufficient DLCs in eels caught in 2007 and 2008 to cause embryotoxicity to medaka (no signs of BSD) which is most likely the case for eel embryos. Thus there was no evident spatial gradient in toxicity along the SLR. Because there was no significant difference among sample sites, there was no evidence for high concentrations of DLCs in the eels collected from even our presumed “positive control sites”, Belgium and Hudson R. These results are in agreement with the chemical analysis of eel tissue, i.e., there appears to be a 100-fold decrease in total TEQs from Kamouraska eels sampled in 1992 (L. Ontario migrants; Hodson et al., 1994) compared to L. Ontario eels from 2007/2008 (Byer, 2009). However, due to the analytical limitations in 1992, the toxic and non toxic congeners could not be separated, which likely resulted in an overestimation of the TEQ values (117-130 pg/g) (Hodson et al., 1994, appendixes 8-12). Byer (2009) re-estimated the TEQs at much lower values of 1-6.7 pg/g, but the method of calculation was not explained in this paper.

There was some variability in the response of medaka to eel extracts with regard to unexpected mortalities at low concentrations and low responses at high TEQs. There were no significant differences among sites for percent normal, hatch or mortality, most likely due to outliers in each location. The differences within these treatments are driven by a small number of fish. There were, however, significant mortalities and reduced hatching in medaka exposed to higher doses of some eels. Eels from R. Sud-Ouest are of particular interest because this location was originally selected as a reference site. However, medaka exposed to 10 eeqs of eel extracts from this site expressed some of the highest percent mortalities (Figure 3-3). This could be explained by one dose having 100% mortality. If this point is taken out, the average percent mortality is much more reasonable, with a value closer to that of the other reference site (Margaree R., Nova Scotia). It is also possible that this site has been contaminated by unmeasured compounds like pesticides, which could have entered the watershed via agricultural application. The mortalities of medaka embryos exposed to high concentrations of eel extracts may indicate that while
dioxin-like contamination may no longer be a problem, there may be other contaminants that still affect embryo survival.

The reduction in maternal tissue contamination implied by these bioassays is mirrored in the chemical monitoring of POPS in L. Ontario sediments and biota such as lake trout and herring gulls (Cook et al., 2003). Estimated TCDD levels in lake trout declined from 200 pg/g in 1967 to 12 pg/g in 1987 (Cook et al., 2003). This reduction in contamination would imply that recruitment of juvenile eels into L. Ontario should be increasing, and there is some evidence of this in the growing number of eels climbing the Moses-Saunders eel ladders at Cornwall, Ontario from 2003 to 2008 (Figure 3-6). Historical counts of eel recruitment into L. Ontario were as high as 1,300,000 in 1982 but declined to lower than 5,000 in 2000 (MNR, 2009). As previously mentioned in Chapter One, the Moses-Saunders hydroelectric dam is operated jointly by the USA and Canada. The Canadian (Saunders) side was the first to install an eel ladder in 1974, and the New York Power Authority later installed one in the Moses dam in 2005.

There are several other possibilities for this recent increase in eel recruitment. The 2003 to 2008 increase in eels ascending the eel ladder correlates to an improvement in oceanic conditions in 1999-2003 (it takes about 5 years for eels to reach the Moses Saunders eel ladder from their breeding ground). From 36 to 57% of the variance in recruitment at Moses Saunders is explained by recent changes in the North Atlantic Oscillation Index and temperatures in the Sargasso Sea (Casselman, 2009). These changes include slightly reduced ocean temperatures and increased current flow, both favourable conditions for eel recruitment. Commercial and recreational management restrictions such as size regulations, trap limitations, and gear restrictions have also been put into effect as of 2005 (Munger et al., 2005), which may also be contributing to the growing number of eels entering L. Ontario. The recent addition of two eel ladders at the Beauharnois dam in 2002 and 2004, could also account for the increasing number of eels climbing the Moses-Saunders eel ladders from 2004-2009 (Table 3-5). These eel ladders would allow for increased passage of juvenile eels up the SLR, artificially increasing the Mosses-Saunders eel ladder index.
Figure 3-7: The number of juvenile yellow eels climbing the eel ladders at the Moses Saunders hydroelectric dam at Cornwall, Ontario (OMNR, 2010).

Table 3-5. Total number of eels ascending both eel ladders at the Beauharnois dam (Bernard and Desrochers, 2007).

<table>
<thead>
<tr>
<th>Year</th>
<th>Number</th>
<th>Size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>10,503</td>
<td>&gt; 420.0</td>
</tr>
<tr>
<td>2003</td>
<td>32,684</td>
<td>&lt; 366</td>
</tr>
<tr>
<td>2004</td>
<td>42,635</td>
<td>350.8</td>
</tr>
<tr>
<td>2005</td>
<td>51,694</td>
<td>344.3</td>
</tr>
<tr>
<td>2006</td>
<td>50,389</td>
<td>349.0</td>
</tr>
<tr>
<td>2007</td>
<td>52,969</td>
<td>360.6</td>
</tr>
</tbody>
</table>
It is also possible that the level of contaminants in eel tissue is underestimated by this bioassay due to the relative insensitivity of medaka embryos to DLCs (Figure 3-8). Unpublished data from our lab on biomarker (EROD) induction place eels close to rainbow trout with regard to dioxin-like sensitivity (Cutler, 2009), assuming that EROD induction predicts dioxin toxicity. According to Elonen et al. (1998), rainbow trout are 3 times more sensitive than medaka and about 4 times less sensitive than lake trout, the most sensitive freshwater fish species yet tested. This may indicate that this medaka bioassay is just at the threshold of detecting the most contaminated eel extracts, which is consistent with elevated mortality rates at 10 eeq, a dose which should compensate for the differences in sensitivity. This raises the question of whether medaka are a suitable surrogate species for American eels? Elonen et al. (1998) argues that fish species with shorter developmental times have a greater tolerance to TCDD, although medaka only take 11 days to hatch, eels take just 4 days (Palstra et al., 2006). This would imply that eel embryos may actually have a higher tolerance for DLCs than medaka, possibly compensating for any insensitivity of medaka to these contaminants. With their year-round egg production, short (11 day) incubation time (although long compared to eels), small egg size, and clear chorion, medaka appear to be the most convenient option as a surrogate species, albeit somewhat conservative with regards to sensitivity.

The reliability of the fish specific toxic equivalency factors (TEFs) used to calculate the 2,3,7,8-TCDD toxic equivalences (TEQs) in this study is another point of uncertainty. TEQs (TEQ = Σ(TEFcongener X concentrationcongener) were calculated using TEFs (TEF = LD50TCDD/LD50congener) established by Van den Berg et al. (1998). The estimated LD50congener was based on potency of induction of EROD, which assumes that the biomarkers’ induction reflects exposure and toxicity; this is a well supported assumption (Safe, 1987; Kennedy et al., 1996; Bosveld, 2002). TEFs are based on the average response of several fish species, the values of which are rounded to 0.5, possibly obscuring variations among treatments. The resulting “fish” TEF may not accurately represent any one tested species, let alone eels which have not been tested.
Figure 3-8: Rank order of sensitivity of 10 freshwater fish species exposed to waterborne TCDD. Medaka are 17 times less sensitive than L. trout (Elonen et al., 1998).
A number of uncertainties have been identified that could compromise the TEF concept when used for risk assessment purposes. These uncertainties include non-additive interactions; differences in shape of dose-response curves, and differences in species responsiveness (Van Den Berg et al., 1998). As well, some penta-chlorinated dioxins (PeCDD) may actually have a higher induction potency in fish than 2,3,7,8-TCDD (Bol et al., 1989; Parrott et al., 1995; Chen and Cooper, 1999). Byer (2009) used Van Den Berg’s (1998) TEF value of 1 for PeCDD to calculate TEQs for this study, but Van den Berg (1998) did not recognize that this compound has a higher potency in fish resulting in TEFs as high as 1.8 (in live trout, Parrott et al., 1995) or 2.6 (in vitro trout liver cell line, Bol et al., 1989). This suggests that the total TEQs in this study may have been underestimated. If the total TEQs are recalculated using a PeCDD TEF of 1.8, a significant ($p=0.004$) average increase of 13% is established, increasing the average TEQ from 1.7 ± 1 to 1.9 ± 1 (n= 25).

The evaluation of toxicity against fish size confirmed that smaller fish are less contaminated than larger fish. Because the river systems closer to the Gulf of St. Lawrence are lower in nutrients than the upper St. Lawrence, the fish residing there have a lower growth rate. The correlation between greater size and higher TEQs suggests that greater contamination of L. Ontario eels is simply a function of greater growth potential, a higher trophic status, and higher biomagnification of POPs at maturity than in other river systems. These river systems were assumed to be less contaminated than the upper St. Lawrence ecosystem, an assumption supported by the one very large fish from R. Sud Ouest that was as low in TEQs as the other smaller eels in this sample (Figure 3-6). Hence it is likely that the TEQs in L. Ontario eels reflect a truly greater degree of contamination, and not simply a difference in growth potential.

While we cannot eliminate a chemical cause for the decline of eel recruitment to L. Ontario, DLCs do not seem to be contributing to the current low rates of recruitment. There are several alternative hypotheses that must be considered, along with the results of this study, to gain a better understanding of this dramatic decline. Thiamine deficiency, turbine mortality, habitat destruction, swim bladder parasites, and over fishing are just a few contributing factors to this complex issue.

**Recommendations**
Conducting isotope analysis of American silver eels to determine where they fed in the L. Ontario food chain would help to determine their trophic similarity to lake trout. This would provide perspective on comparisons between the two species of population status and recruitment success. A study to determine the sensitivity of eels to DLCs in relation to other freshwater fish species (including medaka) would be essential to extrapolate eel embryotoxicity based on surrogate species values. A repeat of these methods using a more sensitive surrogate fish species, such as lake trout eggs, would determine if the current medaka bioassay was sufficient at detecting eel extract embryotoxicity.

**Conclusions**

Mature American eels do not contain enough DLCs in their tissues to be toxic to Japanese medaka embryos and most likely their own offspring. Although the sensitivity of eel embryos is unknown, it is believed that they are more sensitive than medaka to dioxin-like toxicants. If this is the case, medaka may be an inappropriate model for detecting the low levels of contaminants in the eel tissues. Considering the compensating strategy of injecting 10 eeqs, it may still be too soon to declare that chemical contamination is no longer contributing to recruitment decline, because mortalities were still significantly higher in embryos exposed to 10 eeq of eel extracts. Emerging contaminants not tested for in the current study may be causing this response. DCM extractable contaminants such as pesticides, polybrominated diphenyl ethers (PBDEs) (organobromine compounds), PCDEs, DDT, perfluorinated compounds (PFCs) such as perfluorooctane sulfonate (PFOS), chlorobenzene, other non-dioxin like contaminants, and endogenous compounds such as hormones, and vitamins, could be extracted from the eel tissue using the current extraction method and not be detected by the medaka bioassay. This is supported by Martin et al. (2004), who found a significant ($p<0.001$) increase in PFOS concentrations in lake trout in L. Ontario from 1980 to 2001. It is suggested that PFOS is actively transferred from adult females to eggs and that it has implications for early life stage effects (Allsopp et al., 2005). The emerging contaminant theory is also supported by Byer (2010) who found that PBDEs and emerging brominated flame retardants were present in almost all eels sampled from R. Ouelle, QC; Margaree R.,
NS; and Miramichi R., NB. He found that these compounds accumulated in eels to concentrations as high as a mean $68 \pm 29$ ng/g lw ($n=9$) from R. Ouelle, and that they decrease in concentration from west to east. Byer (2010) also determined that these compounds may have declined in eel tissues since 1999 with a roughly 16% decrease in eels from Kamouraska which had a mean $\approx 420$ ng/g lw (Law et al. 2003).

Because of published evidence that past levels of TEQs were higher in L. Ontario and are now decreasing (Cook et al., 2003), a study is currently under way to determine the TEQs in archived eel tissues and compare the toxicity of those eel extracts from 1988 to 2008 using mummichog (*Fundulus heteroclitus*) as the bioassay organism. This study will help to determine if the eels collected in 2007/2008 were at the tail end of the contaminant issue. While these results by no means solve the mystery of the decline of the American eel, they suggest reduction in stress on long-lived predatory fish species of contaminants in the L. Ontario ecosystem.
3.4 References


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Chapter 4 General discussion and summary

4.1 Overview

Chlorinated aromatic hydrocarbons such as PCBs and TCDD/Fs are chemical contaminants commonly found in anthropogenically and industrially-contaminated aquatic environments. Long-lived, fatty fish can accumulate high levels of these contaminants over their lives, especially semelparous fish like the American eel (*Anguilla rostrata*), that only offload their contaminant burden once in their life when they reproduce. The maternal transfer of these contaminants to eggs will likely increase the concentration of toxicants in developing embryos, which are more sensitive than adults to equivalent concentrations of contaminants. This could help to explain the decline of juvenile eels returning to L. Ontario from their birthplace in the Sargasso Sea.

The overall objective of this research was to determine if dioxin-like contaminants (DLCs) accumulated to high enough concentrations in adult American eels while residing in their freshwater habitats were embryotoxic to their offspring. I was also interested in determining if there was a spatial gradient of contamination of eels along the St. Lawrence River (SLR) with L. Ontario being the most contaminated.

The following null hypotheses were tested in this thesis: (1) shaking eggs post injection would not promote the most consistent responses to injected chemicals (rejected); (2) injecting embryos up to seven hours post fertilization would not affect the sensitivity of the egg to the contaminant and its survival (accepted); (3) medaka eggs would not withstand injection volumes > 0.1% of its volume (rejected); (4) there would not be a dose-dependent response of medaka eggs to injected TCDD (rejected); (5) heart rate would not be affected by TCDD (accepted); (6) chemicals accumulated by adult American eels are not toxic to Japanese medaka embryos (accepted); and (7) there is no spatial relationship between toxicity of eel extracts and location along the SLR (accepted).
The present research rejected 3 of 7 null hypotheses; 1, 3, and 4. (1) Shaking eggs post injection not only promoted the most consistent response to injected chemicals, but also reduced mortality and increased hatch synchronicity when reared at 26 ± 1°C; 16 h light: 8 h dark photoperiod. (3) Injecting medaka eggs with 1% of their egg volume (a tenfold increase in recommended injection volume (Walker et al., 1996)) did not cause toxicity and was found to be an accurate representation of 10 eeq when 1 eeq was injected ten times. This allowed for a 10-fold increase in concentration to be delivered when eel tissue extracts could only be concentrated to 1:1. (4) Medaka responded in a dose dependent manner to injected TCDD, making it possible to compare the response of mixtures of chemicals to this bioassay. The accepted null hypotheses were: 2, 5, 6, and 7. The interpretation and significance of the findings are discussed in detail herein.

The exposure method and scoring index were refined specifically for 2,3,7,8-TCDD-injected medaka embryos. The bioassay was optimized for injection time post fertilization, volume tolerance, ideal rearing method and signs of TCDD-like toxicity. (2) It was found that injecting embryos up to seven hours post fertilization would not affect embryo survival or toxicity to TCDD, allowing for more eggs to be injected per day. Medaka typically expressed nine signs of toxicity when exposed to TCDD. The index used to evaluate these signs was tailored specifically for medaka embryos injected with 2,3,7,8-TCDD. (5) Because heart rate was not affected by TCDD in a dose-dependent manner, it was excluded from this index. Measuring heart rate was very time consuming, by excluding it from the index more time could be spent observing the embryos in detail for signs of toxicity.

To test the maternal transfer hypothesis, I compared the toxicity of eel tissue extracts to that of 2,3,7,8-TCDD. I injected newly fertilized medaka eggs with graded doses of TCDD and eel tissue extracts in 1 to 10 nL of triolein using a nano-injector. The eel extracts came from fish collected in 2007/2008 from L. Ontario, R. Ouelle, QC (St. Lawrence estuary); R. Sud-Ouest, QC; Margaree R. NS.; Miramichi R. NB.; Hudson R., NY USA; and Canal Dessel-Schoten, Belgium. Whole eels were homogenized and their lipophilic contaminants were extracted using dichloromethane. Tissue extracts equivalent to 20 ± 1 g (n= 34) were solvent exchanged into injectable triolein. This solution, equivalent to
1 mg of eel tissue/ mg of egg, was serially diluted into the following concentrations 1, 0.5, 0.25, and 0.12 eel equivalents (eeq). A dose of 10 eeq was administered by injecting 10 nL of 1 eeq. Injected eggs were reared on a rotary shaker for twelve days and then scored for signs of toxicity (BSD). The nine signs of BSD that were evaluated were; yolk sac, pericardial and ocular edema, craniofacial and spinal deformities, fin rot, reduced blood circulation, tube heart and body hemorrhaging.

Exposure of early life stages of medaka to a TCDD standard produced signs of blue sac disease (BSD). The ED50 and LD50 of TCDD to medaka with 95% confidence intervals (C.I.) for survival to 12 d post fertilization were 3.79 (1.34-6.39) pg TCDD/mg and 9.57 (6.71 - 13.65) pg TCDD/mg, respectively. (6) However, exposure of medaka embryos to eel tissue extracts did not produce toxicity typical of TCDD exposure, (7) nor a spatial trend in contamination down the SLR system. Not even the eels collected from the presumed “positive control sites” (Belgium and the Hudson R.) caused an increased in BSD in the medaka embryos exposed to their extracts. BSD scores were very low (< 1 out of a possible 15.5), but percent mortality was significantly greater than the controls in some treatments at 10 eeq (p< 0.05), and hatching success and the percentage of normal embryos were significantly lower than those of controls. It is possible that medaka embryos were not sensitive enough to detect the levels of DLCs in the eel tissues considering that eels may be at least twice as sensitive as medaka. Contaminants of emerging concern, non-dioxin-like chemicals, and simply those not selected for analysis in this study, could also be affecting mortality. Any DCM extractable, lipophilic contaminant could be present in the eel extracts, including hormones, vitamins, pesticides, PBDEs, PCDEs, DDT, chlorobenzene, perfluorooctane sulfonate and many more. The toxicants would not be detected by the medaka bioassay because they do not activate the AhR and thus cause BSD in fish. It is also possible that the concentration of DLCs in our freshwater ecosystems have declined to no effect levels. A significant decline in eel TEQs has been observed over the last three decades for fish entering Lake Ontario.

These results indicate:

1. Medaka embryos respond in a dose-dependent manner to injected TCDD, successfully standardizing the TCDD-medaka bioassay.
2. There were not enough TCDD-like compounds in sexually maturing eels collected in 2007/2008 to be embryotoxic to medaka embryos and therefore most likely their own offspring.

3. There was no spatial gradient in toxicity along the St. Lawrence River beginning in Lake Ontario.

4. There is some evidence of non-dioxin like compounds present in eel tissue based on mortality of medaka embryos without BSD.

5. There is a low likelihood that dioxin-like compounds are now limiting the successful recruitment of American eels to L. Ontario.
Appendix A
Preparation of TCDD solutions in triolein

The following procedures were followed to prepare the required dosing solutions for injection into Japanese medaka eggs. This protocol was adapted from similar procedures used at USGS, Columbia, MO, for the injection of various chemicals dissolved in a lipid currier into Japanese medaka eggs.

Solvent exchange

Three micrograms of toluene/TCDD stock solution (50 ng/µL) were transferred into a conical bottomed vial. This vial was placed in a vacuum evaporator (Supelco, 12 port solid phase extraction manifest) under a gentle stream of nitrogen for 15 minutes to evaporate the mixture to dryness, leaving only TCDD behind. The TCDD was re-dissolved in 400 µl of dichloromethane (DCM), then 400 µl of filtered triolein were added; after sonication the vial was replaced under the stream of nitrogen to evaporate off the DCM. The solution was sonicated at 30°C for 5 minutes periodically to ensure dissolution of TCDD in the mixture. This resulted in a stock solution of TCDD in 400 µl of triolein at a concentration of 7.5 pg/nL. A subsample of this solution was sent away for analysis to Environment Canada Burlington, ON to confirm that the concentration was accurate to ± 1 pg/nL (one sample were analyzed three times) and that the subsequent serial dilutions resulted in accurate concentrations. This analysis was conducted using Gas Chromatography- isotope dilution- high resolution Mass spectrometry (GCMS).

After preliminary experimentation, it was determined that a triolein/TCDD solution twice the concentration of 7.5 pg/nL would be required to observe a lethal response in 50% of the embryos. This was accomplished using the identical solvent exchange protocol as above with 120 µL of toluene/TCDD stock solution.

Serial dilution

The following methods are modified from Walker et al., (1996). The triolein/TCDD stock solution was diluted serially in sterile triolein to concentrations of 15, 7.5, 3.75, 1.875, 1.25, and 0.625
pg/nL. The concentrations were selected based on the practical dilution of a limited volume of chemical on the assumption that the medaka specific ED50 for TCDD was 1.572 pg/nL based on a waterborne exposure (Elonen et al., 1998) (Table 1). A DCM control was included by adding 100 µl of filtered triolein to 100 µl DCM; the solution underwent identical procedures as the triolein/TCDD solutions. These solutions were capped while flooding the head space with nitrogen and stored at -20°C.

**Needle filling**

An Eppendorf microlader was placed on the end of a pipette, and filled to 8 µl. The fine tip of the microlader was threaded into the open or back end of the glass injection needle all the way to its constriction. When the microlader tip was fully inserted, the dosing solution was dispensed into the injection needle, making sure not to produce any bubbles. Two needles were filled for each concentration and secured on a strip of plasticine clay in a square Petri dish and stored at room temperature.

**Egg scoring**

Medaka eggs were scored for signs and severity of BSD according to Khan (2007). Unlike previous models, this template accounted for non-treatment mortalities as a separate cause of death and it was tailored to specifically measure responses to 2,3,7,8-TCDD. It also allowed for easy calculation and analysis of BSD score and index with embedded equations in the spreadsheets. This method took a conservative approach to BSD assessment by scoring eggs and hatchlings on the same scale. Eggs were potentially assigned a lower BSD score than hatchlings because of the difficulty of identifying all nine signs of dioxin-like toxicity in an egg. Assessing embryos and hatchlings on the same scale has the added advantage of keeping the BSD index equation the same and keeping all data organized within the same table while not overestimating the response to toxicity.
Table 1. Dose series for practical volumes of dioxin to be injected into Japanese medaka (*Oryzias latipes*) eggs to obtain a response curve which can be related to toxicity in *A. rostrata* embryos.

<table>
<thead>
<tr>
<th>Target Dose pg/g egg wet wt.</th>
<th>Egg wt (mg)</th>
<th>Vol injected (nL)</th>
<th>~ # eggs per g</th>
<th>Target conc. pg TCDD/nL required to hit</th>
<th>Total Vol. of dosing Solution µL</th>
<th>Total pg in Solution TCDD</th>
<th>Practical dilutions</th>
<th>Total Vol. of dosing Solution (µL)</th>
<th>Conc. pg TCDD/nL</th>
<th>Nominal dose pg/g egg wet wt.</th>
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<tbody>
<tr>
<td>0</td>
<td>0.99</td>
<td>1</td>
<td>1010</td>
<td>0</td>
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<td>500</td>
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<td>1010</td>
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<td>1108800</td>
<td>1500000</td>
<td>200</td>
<td>7.5</td>
<td>7576</td>
</tr>
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</table>

# pg TCDD needed
2395800

# µg TCDD needed
2.3958
A score for yolk sac edema (YE) and pericardial edema (PE) was based on the proportion of edema relative to the yolk sac area (modified from Scott et al., 2008). Edematous fluid comprising approximately ¼ of the yolk sac cavity, extending from the ventral surface of the larvae and pushing the yolk to the bottom of the sac was assigned a score of YE 1. Edemas which occupied ½ and > ½ of the yolk sac were represented by YE 2 and YE 3 respectively (Figure 1 b and c).

A score of PE 1 was given to an embryo with edematous fluid surrounding the heart or taking up ¼ of the yolk sac volume; there was usually a bubbling of the pericardial sac close to the jaw. PE 2 represented edematous fluid taking up 2/3 of the yolk volume. A score of PE 3 represented edematous fluid that occupied ½ or > ½ of the yolk sac volume. As pericardial edema increased the heart, which is anchored to the ventral surface of the yolk sac, became stretched at the atrium into a tube-like structure (Figure 1 b and c); heart structure scores typically reflected pericardial edema scores.

Tube heart (TH) was scored on a scale of 0-2 because there are typically two variations in heart formation. A score of TH 1 was given when cardiac looping was impaired so the heart became pulled, resembling an ‘S’ shape, usually associated with PE1 and PE2. A score of TH 2 was given when the heart became fully stretched and resembled a string. At this stage the atrium continued to beat while ventricular beating stopped and blood flow became reduced or ceased. The atrium continued to beat because it is controlled by the brain while the ventricle is controlled by the atrium (Scott, 2008).

The scoring of blood circulation was also on a 0-2 scale and was the most subjective measurement in the present scoring index because absolute values could not be assigned. The score was ranked relative to water controls. Although the atrium continued to beat even at the highest degree of malformation, blood flow velocity was reduced. This led to blockages and regurgitation at the sinus venosus. A score of CIRC 1 was given when the blood flow throughout the entire body was slower than normal; perfusion of blood cells was reduced which may have led to hemorrhaging (score of 0 or 1) in
the caudal fin and yolk sac. A score of CIRC 2 was given when blood flow was much reduced or not present at all; hemorrhaging was almost always found with this circulation score. The embryos were able to survive without blood flow because oxygen diffuses across the chorion and yolk sac and is transported to the organs.

The following signs of toxicity were scored from 0-1 for presence (1) or absence (0). Ocular edema (OE) was scored when edematous fluid surrounded the eye (Figure 1 c). Cranial facial malformations (CF) include blunt nose, cranial protuberances and lower jaw malformations (Figure 1 b, c and d). Fin rot (FR) was defined as the lack of fin rays and the appearance of uncharacteristic circles on the membranous portion of the tail, caudal fin and pectoral fins (Figure 1 b) (Scott, 2008). Spinal malformations may include lordosis (saddle back) of the caudal region or kyphosis (hump back) of the thoracic spine.

Despite the quantitative measurements applied to scoring embryos and fry for BSD, this procedure is not without subjectivity. Because of this, it is important that one person scores an entire experiment, if this is not realistic, than the scoring by two technicians must be calibrated prior to the experiment to elicit the same score per fish.
Figure 1: Images of medaka embryos taken 11 dpf, scale bars represent 1 mm. (a) Normal embryo, straight spine, upturned jaw and absorbed yolk sac. (b) Embryo exposed to 7.5 pg/nL TCDD exhibiting 1) string heart, 2) yolk sac edema, 3) fin rot 4) craniofacial malformations. The arrow indicates jaw malformation and blunt nose. (c) Embryo exposed to 15 pg/nL TCDD with many of the same signs of toxicity as (b); 1) yolk sac edema, 2) ocular edema, and 3) pericardial edema. (d) Prematurely hatched embryo with ruptured yolk sac and severe spinal and craniofacial deformities. Premature hatching was observed in both the 7.5 and 15 pg/nL treatments.
Equation 1: 

\[
BSD\ score = \left[ \sum_{i=1}^{n} (PE \cdot Ei) + \sum_{j=1}^{n} (YE \cdot Ej) + \sum_{k=1}^{n} (CIRC \cdot Ek) + \sum_{l=1}^{n} (TH \cdot EI) + \sum_{m=1}^{n} (SD \cdot Em) + \sum_{o=1}^{n} (CF \cdot Eo) + \sum_{p=1}^{n} (FR \cdot Ep) + \sum_{q=1}^{n} (OE \cdot Eq) + \sum_{r=1}^{n} (BH \cdot Er) \right] \div N
\]

Where \(Ei, Ej, Ek, and El\) are the number of embryos displaying a particular severity of pericardial edema, yolk sac edema, blood circulation, and tube heart respectively and \(Em, Eo, Ep, Eq,\) and \(Er\) are the number of embryos displaying spinal deformities, craniofacial deformities, fin rot, ocular edema, and body hemorrhaging respectively.

The BSD index is the BSD score plus the average mortality score, normalized to the highest possible score (15.5) (Equation 2). Where the BSD index is high due to high rates of mortality, there may be a bias in concluding that the cause was dioxin-like compounds. Hence, the score and index should be considered together.

Equation 2: 

\[
BSD\ index = \frac{BSD\ Score + \sum \frac{Mortality\ score}{N}}{Maximum\ BSD\ score}
\]

A value of 15.5 was assigned for lethality (0.5 higher than the maximum value) and thus the highest possible BSD index (100% mortality) was also 15.5. For example, if four embryos in a treatment of 17 displayed a severity of 3 for pericardial edema, two displayed a severity of 2 for yolk sac edema, six
displayed craniofacial deformity, and two died, then the BSD index would be \[
\frac{(4 \cdot 3) + (3 \cdot 2)+ (6 \cdot 1) + (2 \cdot 15.5)}{17/15.5} = 0.229.
\] See Table 2 for a sample scoring chart of BSD score and index.

References


Table 2. Sample scoring chart for embryo and larva BSD at 11 dpf. A score of 15.5 was assigned to embryos that died with severe BSD. Both embryos and hatched larvae were scored using the same scoring criteria. 0.31 would be the reported BSD index for this treatment.

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<tr>
<th>Treatment</th>
<th>YS</th>
<th>PE</th>
<th>OE</th>
<th>TH</th>
<th>CIRC</th>
<th>BH</th>
<th>CF</th>
<th>SD</th>
<th>FR</th>
<th>BSD Score</th>
<th>Dead</th>
<th>BDS Index</th>
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<tr>
<td>Max score</td>
<td>(3)</td>
<td>(3)</td>
<td>(1)</td>
<td>(2)</td>
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<td>(1)</td>
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<td>0.25 eeq N=15</td>
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<td>0.09</td>
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<td>0</td>
<td>0.31</td>
<td>-</td>
<td>(4.73/15.5)</td>
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Appendix B

Preparation of embryo rearing solution in stock aliquots

Embryo rearing solution was prepared by making stocks of four salts, and adding the stocks together. This solution was enough to make nine 50 mL aliquots of 20 times concentrated stock (9 L total of rearing solution). The following four stocks were prepared by adding the proper amount of each salt to 100 mL of deionized water in an Erlenmeyer flask. A stir bar was used to spin the salt into solution.

<table>
<thead>
<tr>
<th>Stock</th>
<th>Preparation</th>
</tr>
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<tbody>
<tr>
<td>1. 10% NaCl:</td>
<td>10 g NaCl in 100 mL dH₂O</td>
</tr>
<tr>
<td>2. 0.4% CaCl₂ · 2 H₂O:</td>
<td>0.4 g CaCl₂ · 2 H₂O in 100 mL dH₂O</td>
</tr>
<tr>
<td>3. 0.3% KCl:</td>
<td>0.3 g KCl in 100 mL dH₂O</td>
</tr>
<tr>
<td>4. 1.63% MgSO₄ · 7 H₂O:</td>
<td>1.63 g MgSO₄ · 7 H₂O in 100 mL dH₂O</td>
</tr>
</tbody>
</table>

The above 100 mL solutions were added to a 500 mL Erlenmeyer flask, an extra 100 mL of dH₂O were added (total volume should be 500 mL). The stock solution was split into nine 50 mL aliquots using a graduated cylinder and stored in conical test tubes at 4°C. When ready to be used, a test tube of concentrated ERS was removed from the fridge and left out overnight to bring the solution up to room temperature. The 50 mL concentrated ERS solution was then poured into a 1 L flask and 950 mL dechlorinated water and one drop of methylene blue mold inhibitor (which will turn the solution light blue) was added (Modified from Glase and Reed, 2000).

References
Appendix C

Standardizing the TCDD Bioassay

The Japanese medaka (*Oryzias latipes*) bioassay was standardised to achieve reliable ED50 and LD50 values for injected 2,3,7,8-TCDD (TCDD). TCDD is potentially the most toxic halogenated aromatic hydrocarbon to fish (Van den Berg *et al.*, 1998), and as such, serves as the basis of the current bioassay calibration study. This bioassay was also standardized in preparation for injection of fish tissue extracts, specifically American eel (*Anguilla rostrata*) tissue. In early life stages of fish, exposure to TCDD produces signs of toxicity (blue sac disease; BSD) that include; yolk sac, pericardial, and ocular edema, body haemorrhaging, tube heart, reduced circulation, fin rot, spinal deformities, and craniofacial malformations (which include jaw deformities and cranial protuberances). These nine signs of toxicity are specifically expressed in medaka embryos injected with 2,3,7,8-TCDD congener. Lack of hatch and mortality were also included in assessing toxicity.

Eggs were injected between 2 and 3 hours post fertilization to simulate maternal transfer of contaminants. The bioassay had a fixed exposure time of 11 days post injection (day 0 was the day of egg fertilization). This end point was selected based on the time to hatch at 26 ± 1°C, in an 18 h light: 6 h dark cycle, reared on a platform shaker (which synchronized and reduced hatch time). On day 11 the medaka (either eggs or fry) were scored for signs and severity of TCDD toxicity. With this method of scoring came experimental bias based on the assumption that all signs can be seen equally in eggs as in fry. Due to being coiled upon itself in the egg, it is very difficult to score fin rot, spinal deformities, and ocular edema in un-hatched embryos. This can be seen in figure 1B, where the frequency of these signs’ occurrences falls in the last two concentrations when lack of hatch increases above 69%. Compared to the signs which are easily scored in eggs, a dose response relationship is much clearer (Figure 1A).

Craniofacial malformations were not included in this comparison because the score for this sign was
elevated in all treatments. Because of the scoring discrepancy, eggs should be scored on a separate scale than hatchlings, however to keep the scoring template simple, hatchlings and eggs were scored together. To determine the accuracy of scoring toxicity in eggs versus hatchlings, eggs were scored on day 11 and then allowed to develop for 3 more days and scored again as hatchlings. There was no significant difference ($p=0.48$) between the two scores suggesting that the results of this combined approach would yield an accurate yet conservative score of toxicity.

Mortality before day 11 also obscures the true BSD score because individual signs of toxicity are not scored, rather the embryos receive a maximum score equivalent to all of the signs of toxicity plus 0.5 (15.5 in this case). To compensate for this, mortalities are included with BSD scores in a separate calculation called BSD index. This allows the researcher to consider BSD score independently of mortality and identify that mortality may not be caused by BSD pathology.
Figure 1: Average occurrence of individual signs of toxicity (the highest score of an individual sign is 3) caused by TCDD exposure for (A) the five signs clearly observed in embryos and (B) the four signs most difficult to observe in embryos.
Determining the excretion of dosing solutions from medaka eggs post injection

After injecting medaka eggs with TCDD or fish tissue extract, it is important to determine if the entire dose is absorbed or if some is excreted by the embryo. Excretion of the dosing solution could bias the final toxicity score. Visual observation of the embryo during and after injection determined that no fluid leaked out of the injection site. To confirm this observation, medaka eggs were injected with 1 nL of 3.84 µg/mL of the fluorescent label pyrromethene 546 dissolved in triolein. The embryos and their surrounding ERS were then viewed under a confocal microscope at an excitation and emission wavelength of 492 nm and 500 nm respectively (Figure 2A-D). The integrated optical density (IOD) of the pyrromethene was measured at 1 hour post injection (hpi) and then again at 7 days post injection (dpi) using a confocal microscope and spectrophotometer. IOD is a fluorescence measure of the concentration of the injected pyrromethene. There was no significant difference (p= 0.24) between the IOD of the 1 hpi and 7 dpi embryos, indicating there was no substantial loss of fluorescence. The confocal imaging also determined there was no pyrromethene in the surrounding ERS at 1 hour or 7 days post injection. These results were supported by Zabel et al., (1995) who found that rainbow trout eggs retained >90% of injected radiolabeled compounds through to hatch. An added benefit of injecting the fluorescent label was that I was able to identify what tissues were destined to be affected by the injection solution by 7 dpi (yolk, spleen, pigment cells).

Average ED50

The BSD index results for four replicates of the TCDD injection bioassay were graphed (Figure 3). The resulting ED50s for each sigmoidal dose-response curve were averaged and the 95% C.I. were calculated to establish 4.18 ± 0.9 pg/mg (average n= 64) as the 11 day ED50. This method of estimating the ED50 shows differences among the reps in the distribution of data points at each dose, but overall a high degree of repeatability.
Figure 2: Confocal imaging of embryos injected with the fluorescent label pyrromethene 546 at 1 hpi (B) and 7 dpi (C and D). Image (A) is the control with no pyrromethene injected.
Figure 3. The average ED50 to express BSD for four repetitions of TCDD injected embryos was 4.18 ± 0.9 pg/mg (average n= 64).

References

Safe, S. 1990. Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Crit. Rev. Toxicol. 21: 51-88
Appendix D

Eel extract preparation and quality assurance

Eels were collected in 2007 and 2008 throughout eastern Canada, and stored frozen at -20°C. Eels were homogenized and stored in glass jars as 20 ±1g (n= 34) aliquots at -80°C at the National Biological Tissue Archive at Environment Canada, Burlington, Ontario.

Part 1: Protocol from J. Byer

The following is a summary of the methods refined by Jonathan Byer, department of chemistry, Queen’s University, Kingston, Ontario. Details of this procedure can be obtained from Jonathan directly. This method only covers the extraction and cleanup portion of the testing procedures and is applicable to the following fish only; Margaree R., NS eels 2,4,5,6,7; Miramichi R., NB eels 3,5,6,7,8, and R. Ouelle, QC eels 2,3,5,7.

Sample preparation

Eel tissue extracts were prepared for chemical analysis from approximately 20 g of homogenate. Each sample was dried chemically with anhydrous Na₂SO₄. The mixture was stirred frequently until it was dry and free flowing, and spiked with 2,2’,3,3’,4,4’,5-[¹³C₁₂]-HeptaCB and 2,3,7,8-[³⁷Cl₄]-TCDD.

Extraction

The extraction columns were prepared by inserting a small glass wool plug into the bottom of each chromatography column. The sample mixture was then poured into the column followed by 50 mL of dichloromethane (DCM). Elution continued at a rate of 5-10 mL/min with a total of 350 mL DCM. The extract was split by weight into four portions; one fraction was spiked with a solution of fifteen ¹³C₁₂-labelled PCDD/F surrogates and four ¹³C₁₂-labelled coplanar PCB surrogates.
**Extract concentration**

The eluant was concentrated to 60 mL by rotary evaporation at 40°C then quantitatively transferred to 10 graduated tubes (6 mL each)

**Lipid removal**

Each 6 mL aliquot was run through gel permeation chromatography (GPC) with Biobeads SX-3, and a 2-layered packed 5% deactivated silica-alumina column for bulk lipid cleanup. A spiked sample was run through every few samples as a quality control step to estimate percent recover (usually around 90%). The dLPCBs and PCDD/Fs were separated on a Cosmosil 5PYE column by high performance liquid chromatography. The two fractions were reduced in volume and spiked with additional $^{13}$C$_{12}$-labelled PCDD and PCB surrogates used as instrument standards. The samples were recombined and concentrated to 1 mL by rotary evaporation. Each sample was then passed through silica gel with hexane to remove any remaining lipid. The samples were then made-up to their final volume of 10 mL with hexane and stored in glass vials until analysis.

**Part 2: Protocol from ASU**

The following methods were prepared by Alison Rutter the director of the Analytical Service Unit, Queen’s University, Kingston, Ontario. This method is applicable to the following fish only: R. Sud-Ouest, QC eels 1,2,3,4,5; L. Ontario, ON eels 2,3,4,8,9; Hudson R., NY eels 1,2,3,7,8; Canal-Dessel Schoten, Belgium eels 1,2,3,4,6. Five jars per eel were sent to Queen’s University for analysis (approximately 100 g).

Materials:

- 1 jar of eel homogenate
- 1 porcelain mortar, 750 mL capacity and pestle
- Anhydrous sodium sulphate
- Ottawa sand
Each homogenate sample was:

1. Removed from storage and thawed, mixed thoroughly to recombine any separated lipid with the tissue. Jar information was documented.

2. All 100±1 g of the eel homogenate was, weighed in a solvent-rinsed aluminum boat, quantitatively into a large (750 mL) mortar with 200 g (2 x 100) of anhydrous Na₂SO₄ and 5 to 6 scoops of Ottawa sand.

3. The sample mixture was ground manually until it was a free-flowing mixture (dry).

4. The mixture was transferred quantitatively with rinses of DCM into a large Soxlet column (22 mm I.D. x 500 mm length) plugged with silanized glass wool,. Let run for six hours.

5. The balance of the DCM was eluted from an extraction reservoir such that 350 mL of solvent was passed through the column at a rate of 5-10 mL/min. The eluted solvent was collected in a pre-weighted 500 mL round bottom flask.

6. Steps 1-5 were repeated for the remaining 4 jars of eel extract.

7. Each fraction was reduced in volume to a few mLs by rotary evaporation. Using a graduated cylinder, 10 fractions of 6 mLs each was prepared.

8. DCM extracts the lipophilic contaminants from the eel tissue along with the tissue lipids. Lipids were removed by gel permeation chromatography (GPC) of the extracts, split into 10 fractions (10 hours).
9. After GPC, 60 mL of eel has been reduced to 50 mL (10 mL loss). Once the lipid was removed the fractions were recombined and concentrated by rotary evaporation and solvent exchange into hexane.

10. A final clean up step removed any remaining lipid. Ten mL of extract was poured into 5 silica columns and rinsed with hexane. The remaining solutions were recombined and subdivided into 5 vials. Each vial represents 1 soxhlet and 20 ± 1 g of eel tissue.

Quality Assurance

Column chromatography mass spectrometry (GC/MS) was used to test spiked samples for accuracy of lipid extraction and contaminant recovery. Columns of eel samples were spiked with 50 µL of 2 ppm surrogate internal standard $^{13}$C$_{12}$-CB (170) and 2,37,8-(37Cl$_4$)-TCDD. It was determined that 90-100% of contaminants were recovered using the Soxhlet and GPC protocol.
Appendix E Total TEQs

The total TEQ data (Table 1) used in this study were obtained through personal communication with Jonathan Byer, Queen’s University department of Chemistry. These data were acquired through chemical analysis of eel tissue to determine the concentration of contaminants in each eel, using the TEF values from Van den Berg et al. (1998), and these concentrations, the TEQs were calculated.

Table 1. Total toxic equivalents (TEQ) measured from each eel collected from five sample sites along the St. Lawrence River System. Total TEQs are comprised of total PCDDs, total PCDFs, and total dioxin-like PCBs. TEQs were calculated based on the Toxic Equivalent Factors (TEFs) from Van den Berg et al. (1998).

<table>
<thead>
<tr>
<th>Fish No./ Site</th>
<th>Margaree R.</th>
<th>Miramichi R.</th>
<th>R. Ouelle</th>
<th>L. Ontario</th>
<th>R. Sud-Ouest</th>
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</table>

Reference

Appendix F

Reassessment Validation of BSD index for eel extract injections

Variability in the response of medaka embryos to injected eel extracts could be seen in their unusually high BSD index at relatively low eel extract concentration (Figure 1). These responses did not follow a predictable dose response curve, prompting a repeat of 12 unusual data points selected from Lake Ontario, Margaree R., NS., R. Sud-Ouest, QC., R. Ouelle, QC., and the Hudson R. U.S.A.

Newly sharpened injection needles were filled with eel extracts previously prepared for toxicity bioassays. The experiment was repeated for the 12 unusual points which resulted in lower mortality for 11 of the 12 points but an increase in mortality for L. Ontario 8 eel at 1 eeq (circled points in Figure 1). This indicates that for the most part, the negative response was validated with repetition, confirming that eel tissue extracts did not induce toxic responses in medaka embryos. The data from these repeated points were used in this thesis while the unusual data points were discarded. The re-graphed BSD index data can be found in Figure 2.
Figure 1. (A) BSD index for individual eel extracts with unusual values (average n= 16). (B) Resulting BSD index for eel extracts after they were re-injected using new needles. The circled points in the L. Ontario figure indicate that these values actually reversed after repetition.
Figure 2. BSD index for individual eels from all seven locations (average n= 16) after the 12 unusual treatment points were repeated.