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Toxicology of Decabromodiphenyl Ether in Avian Embryos: Disposition of the Flame Retardant BDE-209 in Yolk-Injected Chicken Embryos (Gallus gallus)

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Toxicology of Decabromodiphenyl Ether in Avian Embryos: Disposition of the Flame Retardant BDE-209 in Yolk-injected Chicken Embryos (*Gallus gallus*)

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Samantha D. Sifleet

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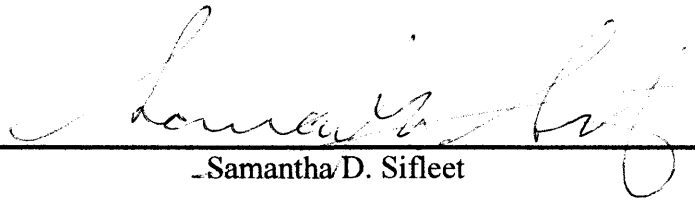

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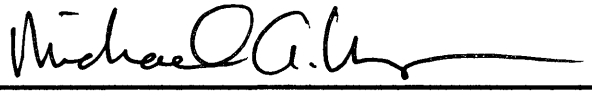

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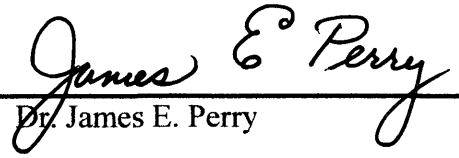

Dr. James E. Perry

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ABSTRACT

Polybrominated diphenyl ethers (PBDEs) are flame retardant chemicals of toxicological concern present in humans, wildlife, and the environment. Deca-BDE is the highest production product due to historical use patterns and recent regulatory limitations on the other two commercial formulations (Penta-BDE and Octa-BDE) in the U.S and Europe. The EU banned Deca-BDE starting July 1, 2008. However, it remains in usage in North America and elsewhere in the world. BDE-209 is the dominant congener in all Deca-BDE commercial products. BDE-209 has been reported to under go metabolic debromination to lesser brominated and more toxic and bioaccumulative congeners. However, insufficient data are available on this process. It has also been observed that congener profiles and BDE-209 levels in terrestrial organisms differ from most aquatic species, indicating accumulation or metabolic dissimilarity. The goal of this *in ovo* study is to determine the biotransformation and tissue distribution of BDE-209 after injection into the yolk-sac of embryonic chickens. An emulsion formulation was employed to better distribute the hydrophobic BDE-209 within the eggs in an attempt to better mimic "natural" exposure of embryos. Acute mortality from BDE-209 yolk injection was observed. An LD50 value of 44 μg/egg (740 ng/g ww) was determined for embryonic chickens in this study. Concentrations of BDE-209 and possible metabolic degradates were determined in five compartments of the embryos (yolk, brain, liver, heart and remaining carcass). The results indicated that some BDE-209 was mobilized from the yolk, into the carcass, liver, brain, and heart tissues of the developing chicken embryo prior to pipping. However, 80% of the dose was detected as BDE-209 in the yolk sac. Additional BDE-209 would likely have been assimilated following hatching and resorption of the remaining yolk. Nona-BDEs were detected in all of the liver and yolk samples from BDE-209 exposed eggs. The congener profiles of the different tissues did not indicate that significant metabolic debromination of BDE-209 occurred within the developing embryos.

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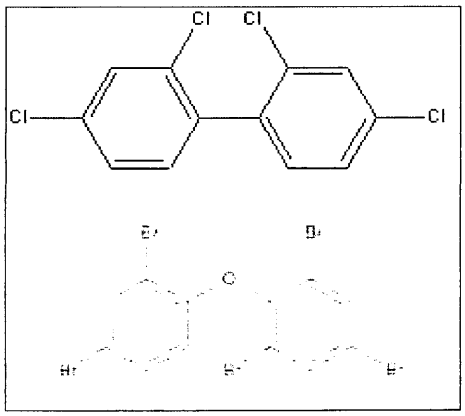
INTRODUCTION

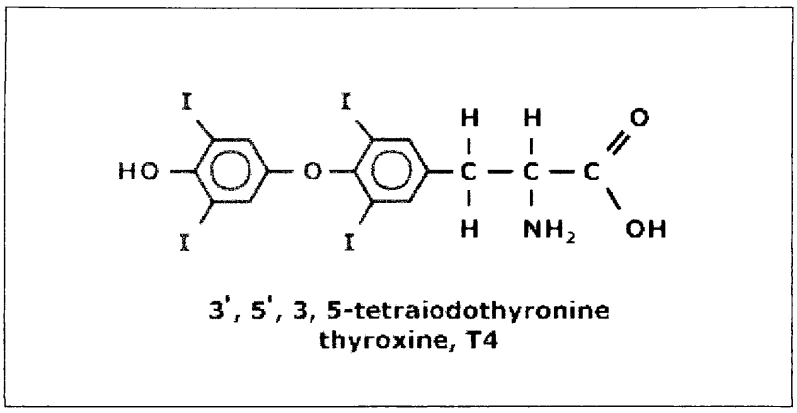
Polybrominated diphenyl ethers (PBDEs) are flame retardant chemicals present in many industrial and household products. PBDEs have been widely used in thermoplastics, polyurethane foam and in textile coatings (Watanabe and Sakai 2003, Hale et al. 2003, Law et al. 2006). The chemical structures of PBDEs are reminiscent of polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs) (Figure I.1). There are a total of 209 individual PBDE congeners possible, as is the case for PCBs and PBBs, but a limited number are abundant in the commercial mixtures (LaGuardia et al. 2006). The ether linkage between the two phenyl rings in PBDEs, and the presence of bromines versus chlorines, results in these molecules bearing a closer resemblance to natural hormone molecules such as iodine-containing thyroxine than the PCBs (Figure I.2).

Figure I.1: PCB versus PBDE



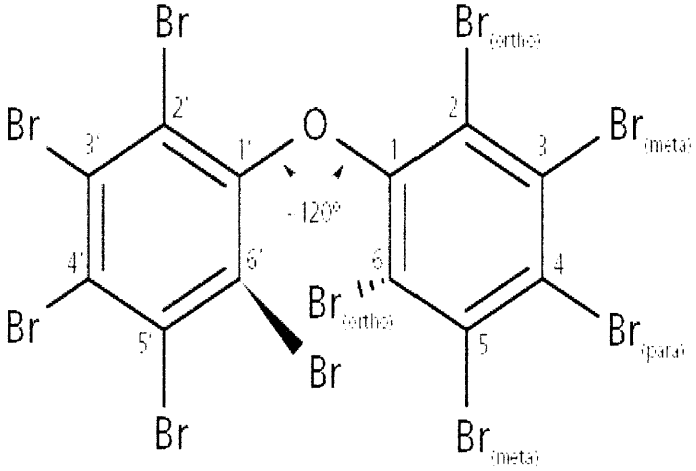
The top molecule is a polychlorinated biphenyl (PCB) congener; specifically 2.2',4.4'-tetrachlorobiphenyl (PCB-47) . The bottom image is the molecular structure of the polybrominated biphenyl ether (PBDE) congener (BDE-100). (www.slh.wisc.edu/ ehd/organics/cleanroom.php)

Figure I.2: Thyroxine, T4



Three PBDE commercial mixtures have been in wide commercial use since the 1970s. These products vary in regards to the degree of bromination of their constituent congeners. Penta-BDE is the least brominated mixture. Its dominant constituents exhibit from three to six bromines. Octa-BDE contains components that average eight bromine substitutions, but major congeners exhibit six to nine substitutions. Deca-BDE consists of about 97% of the fully brominated BDE-209 congener (Figure I.3).

Figure I.3: Decabromodiphenyl ether, BDE-209



The commercial PBDE mixtures are used in different polymer products. The bulk of Penta-BDE has been employed as an additive in polyurethane foams, common in

furniture. It appears that Penta-BDE was also used to a limited extent in some circuit boards until the mid 1990s. Octa-BDE was a more modest use product, mostly as a flame retardant additive in acrylonitrile-butadiene-styrene (ABS). ABS is a high-impact plastic used in office electronics, automobiles and kitchen appliances. (Hale et al. 2003, Watanabe and Sakai 2003)

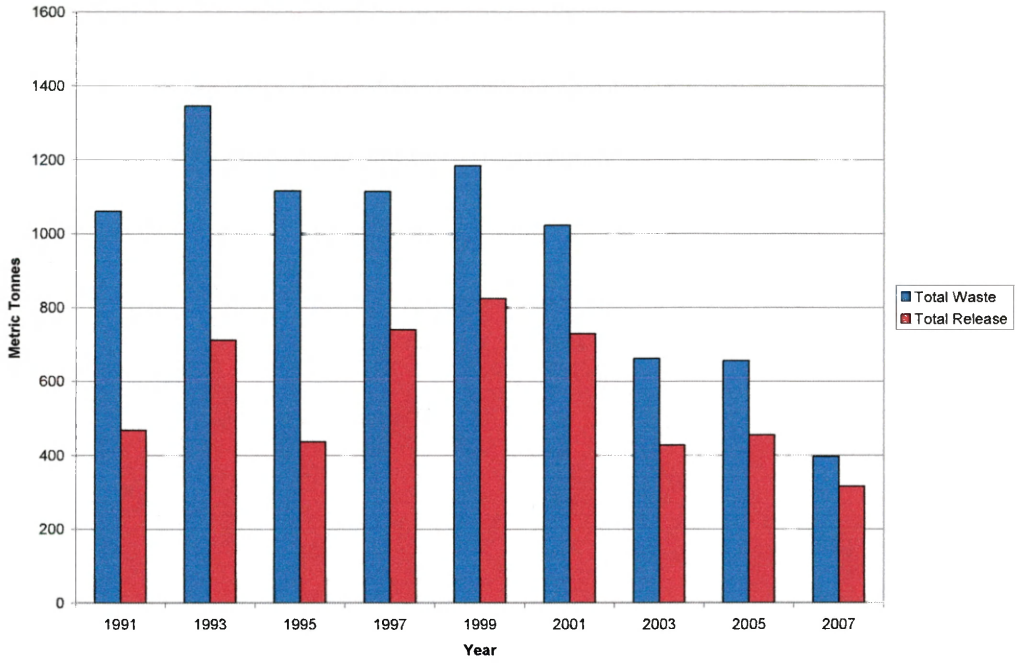
In 2001, Deca-BDE constituted 83% of total global PBDE use (Hale et. al 2003), primarily as an additive to high-impact polystyrene[1]. This plastic is commonly used in the housings of various electronic devices such as televisions, computers, and related products. Deca-BDE is also used in latex back-coatings for textiles. Polymer products can contain up to 30% Deca –BDE by weight. With the cessation of the U.S. production of Penta-BDE and Octa-BDE in December 2004, Deca-BDE remained as the sole commercial PBDE product on the North American market.

Deca-BDE is listed as a toxic, high production volume chemical by the US EPA. As such, it is subject to annual release reporting by US industries using this chemical. The Toxic Release Inventory Program (TRI) maintains a database on chemical release documentation going back to 1988. The TRI was originally created under Section 313 of the Emergency Planning and Community Right to Know Act (EPCRA). The Pollution Prevention Act of 1990 expanded TRI to pursue data on source reduction as well as release information. These data are available on-line at the EPA website.[2] Following the methods from Hale et al. (2006) a brief analysis of these data was run to update the dataset of Deca-BDE releases to the environment over the last two decades. The data from the TRI are accumulated on a voluntary basis, therefore some considerations of the

[1] http://www.bsef.com/uploads/library/BSEF_factsheet_Deca-BDE_160108.pdf
[2] http://www.epa.gov/TRI/

data quality can not be assured. For more information on this matter the EPA has released a guidance Document entitled "The Toxics Release Inventory (TRI) and Factors to Consider When Using TRI Data."

Figure I.4: Deca-BDE waste and release figures from the EPA Toxic Release Inventory



The global demand for Deca-BDE in 1999 was 54,800 metric tons. (Watanabe and Sakai, 2003) The American market represented 44% of total global demand, the Asian 42%, and the European consumption only 14%. Total DecaBDE in US waste for 1999 was 1185 metric tons or 2% of the global demand. Law et al. 2006 reported the estimated annual production of Deca-BDE to be 30,000 metric tones worldwide.

Hale et al (2009) examined temporal trends of PBDE concentrations in Chicago biosolids (stabilized sewage sludge) from the 1970s to 2008. The sludge samples showed a parabolic trend for the Penta-BDE product over time, with peak concentrations around

the year 2002 and then a decrease (Hale et al. 2009.) This finding is consistent with the end of Penta-BDE production in 2004. The Deca-BDE concentrations in the Chicago sewage sludge increased over time. Continued release overtime and high persistence would support such a trend. Gerecke et al. (2006) found BDE209 to have a half-life of 700 days in anaerobic sewage sludge.

I.1: PBDE Timeline

1970s Manufacturing of PBDEs begins. (Hale et al. 2006)

1981 PBDEs first detected in a river downstream from a textile plant in Stockholm.

2004 The European Union (EU) bans the Penta-BDE and Octa-BDE formulations. (Kierkegaard et al. 2009)

2004 U.S. PBDE manufacturers, Abermarle Chemical and Great Lakes Chemical, enter into an agreement with the U.S. EPA to cease production of the Penta-BDE and Octa-BDE products.

2005 The EU grants a four-year postponement of the ban on Deca-BDE.

2007 Maine and Washington States ban the use of Deca-BDE in electronics and new mattresses and other furnishings (Kierkegaard, 2009)

2008 The EU regulates all uses of Deca-BDE through the Registration Evaluation Authorization of Chemicals (REACH) Program.[3]

There are regulatory differences between the U.S. and Europe with regard to PBDEs. Most usage of Penta-BDE and Octa-BDE ceased in the EU due to environmental concerns, well before the formal 2004 ban . In the United States their usage continued until their sole manufacturer, Great Lakes Chemical Company, voluntarily entered into an agreement with the U.S. EPA to end production by December 2004. Deca-BDE is now believed to be the only PBDE product remaining on the market in the US and abroad. But its safety remains a controversial issue. Deca-BDE is an effective flame retardant and a high production volume chemical, generating significant profits for its manufacturers.

PBDEs have been identified and quantified in many types of environmental and human samples and concentrations have risen therein since the 1970s (Hites 2004). Burdens in US samples tend to be higher than in European ones, most likely due to earlier regulatory actions taken in Europe..

Deca-BDE is a persistent organic pollutant. The major BFR manufacturers have historically claimed that Deca-BDE does not bioaccumulate and has extremely low bioavailability. Levels in aquatic organisms are typically much lower than the less brominated congeners. However, recent research has reported that BDE –209 was disproportionately high versus other PBDE congeners in some terrestrial organisms, e.g. grizzly bears (Christensen et al. 2005), red foxes (Voorspoels, 2007) and selected bird species (Lindberg et al. 2004, Chen et al. 2007, Potter et al. 2009). The levels of BDE-

[3] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2008:131:0007:0012:EN:PDF

209 in terrestrial organisms may be linked with the proximity of the food web to point sources of PBDE pollution. For example, (Potter et al. (2009) reported that BDE-209 levels in peregrine falcons (*Falco peregrinus*) from the Chesapeake Bay region were significantly correlated with population densities.

I.2: Presence of BDE-209 in Birds and the Environment

The fate and persistence of organic compounds is directly related to their structure and physical-chemical properties. BDE-209 has an octanol/water partitioning coefficient (log K_{ow}) of 8.70. (Waina and Dugani, 2003) This high value equates with the compound being very hydrophobic. The molecular size of BDE-209 and modest lipophilicity led many to believe it would not bioaccumulate. However, the literature shows that BDE-209 is detectable in humans, mammals, birds, soils, plants, sewage sludge, and indoor dust (Voorspoels et al. 2007, Christensen et al. 2005, Hale at al. 2003, Law et al. 2008).

In 2008, Law et al. reviewed published data on brominated flame retardants (BFRs) in the European and Asian environments, particularly the concentrations and trends of BDE209 in biotic and abiotic media. The increasing trend in Europe appeared to be a leveling off. While, BDE209 concentrations continued to increase in Asian environments. The leading theory behind this is that the prevalence of electronics recycling in Asia results in large releases of BDE209 into the environment. Law et al. (2008) also highlighted the difficulties in analyzing samples for BDE209, in part due to its low volatility and thermal instability.

Christensen et al. (2005) analyzed fat tissue of grizzly bears from British Columbia and reported significant differences in the congener profiles of bears feeding in a maritime versus a terrestrial environment. The differences in diet were determined using stable isotope analysis. Enriched $\delta^{13}C$ values indicated more marine food items in the diet, while enriched $\delta^{15}N$ values indicated higher trophic prey items. Total PBDEs detected were not statistically different between the two groups. However, the congener profiles between the two were remarkably different. The maritime bears contained a preponderance of lower brominated congeners, i.e. BDE-47>209>99>100>153. The profiles of inland bears were dominated by more heavily brominated congeners 209>47>207>208.

Another study examining moss samples (Mariussen et al., 2005) further demonstrated that BDE-209 is capable of entering the terrestrial food web. Concentrations of BDE-209 in moss samples from Norway ranged from 0.11 to 1.59 μg/kg dw. BDE209 was the dominant congener therein, 85% of the total PBDEs. Sellstrom et al. (2005) examined uptake of PBDEs by earthworms from sewage sludge-treated soils and reported that bioaccumulation factors decreased with molecular size. In 2007, Voorspoels et al. analyzed PBDEs in tissues of the terrestrial red fox (*Vulpes vulpes*.) and found BDE209 was a dominant congener in the tissue profiles, representing 70% of the total PBDEs.

While limited studies are available, burdens of the more brominated PBDE congeners have been observed to be remarkably higher in some predatory bird species. For example, a study of peregrine falcon eggs in Sweden detected BDE-209 in 18 of 21 egg samples. The mean concentration values were 130 ng/g lipid in the southern

population and 110 ng/g in the northern population (Lindberg et al., 2004). Another European study (Herzke et al., 2005) examined eggs from six species of Norwegian predatory birds. The species were evenly split between marine and terrestrial feeding specialists. Herzke et al. (2005) did not report BDE-209 in any of a total of 62 hatched eggs. However, BDE-183 was quantifiable only in peregrine falcon eggs and was consistent with the findings of Lindberg et al. (2004). Furthermore, Herzke et al., 2005 documented species dependent PBDE congener patterns. Jaspers at al. (2006) evaluated oganohalogen body burdens in seven species of aquatic and terrestrial predatory birds from Belgium. . In this analysis BDE-209 and BDE-183 were only detected in the terrestrial birds.

Liang et al. (2008) identified BDE-209 as the dominant PBDE congener in foraging chicken hens from an electronic waste recycling area in southern China. This study showed peak BDE-209 levels in muscle tissue of 17,977 ng/g lipid wt. Chen et al. (2007) detected BDE-209 in birds of prey from northern China. Specifically, BDE-209 was detected in 79.4% of the analyzed samples. Furthermore, it was the dominant congener in samples from some buzzards, scops owls, and long-eared owls. The highest liver levels of BDE-209 were detected in common kestrels, with a mean of 2,870 and maximum of 12,200 ng/g lipid weight. These values are much higher than those previously reported for BDE-209. The authors postulated that the dominance of the BDE-209 congener in these samples may be related to the large scale production, usage, and disposal (recycling of obsolete imported and domestic electronics) of Deca-BDE containing products in China.

Luo et al. (2009) examined the presence of PBDEs in free-range domestic fowl within an electronic-waste recycling site in South China. Male and female chickens (*Gallus domesticus*) and ducks (*Anas platyrhynchos domesticus*) were examined. Samples of muscle and liver tissue from 51 birds were analyzed for PBDEs. The BDE-209 levels were higher in chicken (14-25 ng/g lipid wt.) than ducks (2.1-3.9 ng/g lipid wt.). While both species eat grain (9.7 ng/g dry wt.), the chickens also foraged for insects on land and may have been exposed to additional BDE-209 via consumption of soil and invertebrate prey.

In the USA, relationships between human population densities and PBDE congener patterns in wild birds have been identified. Potter et al., (2009) examined peregrine falcon eggs from the Chesapeake Bay region. BDE-209 levels were found to be significantly higher in birds nesting in densely populated urban areas. The levels of the octa and nona BDE congeners in these samples typically exceeded the BDE-209 levels. While the reason for this is not fully understood, the authors postulated this was due to metabolic debromination of BDE-209 within the birds.

I.3: Toxicology and Pharmacology of PBDEs

The US EPA released a Toxicological Review of BDE-209 in June of 2008. This document is a comprehensive review of the available data on the toxicology of BDE-209. However, limited data were available for avian species. Therefore, the scope of this section includes a discussion of the toxicology of all PBDEs in a wide variety of species. Only dose-response relationships are discussed.

Traditional feeding studies that exposed mammals to BDE-209 dissolved in a solvent carrier show uptake rates of only 0.1-5% (Norris et al., 1975; El Dareer et al., 1987). In feeding study of rats using radiolabeled ^{14}C Deca-BDE (Norris et al., 1975) assimilation of the dose was low and complete elimination of the isotope was observed within two days. In contrast, retention of the Octa-BDE product was up to 40% within the rat. Norris et al. (1975) established a no observed effect level (NOEL) for Deca-BDE in rats of 8 mg/kg body weight per day in a 30 day dosing trial. Another study showed an uptake efficiency of 0.025–0.55% of Deca-BDE via dietary exposure over 14 days (El Dareer et al., 1987).

To mimic stomach contents and enhance the concentration of Deca-BDE accumulated, Morck et al. (2003) utilized an emulsion-based delivery vehicle, as is often used in drug delivery studies. It allows for increased suspension of large organic molecules in water. This approach may act as a more realistic delivery vehicle for dietary exposure of BDE-209 and permit heightened detection of candidate breakdown products. The emulsion delivery vehicle (Morck et al., 2003) for Deca-BDE resulted in a 10% or greater absorption of BDE-209 into the rat.

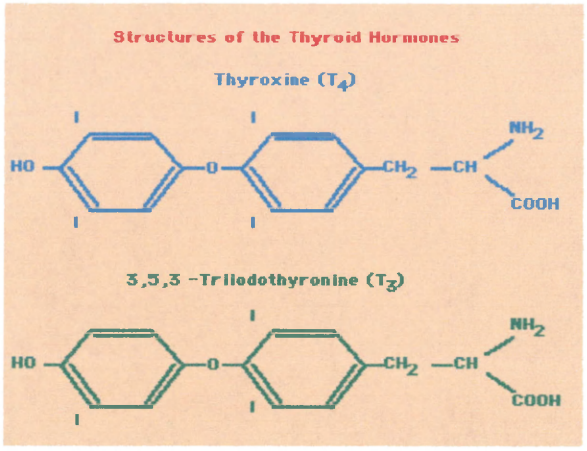
In a combined gavage and intravenous exposure study, Sandholm et al. (2003) showed an absorption rate of 26% in the rat. This study used an emulsion carrier similar to Morck et al. (2003). A total of 13 phenolic metabolic breakdown products were identified. This lends further support to the oxidative mechanism proposed by Morck et al. (2003).

In a feeding exposure and depuration study, Stapleton et al. (2004) observed no net accumulation of BDE-209 in juvenile carp (*Cyprinus carpio).* However, seven

different compounds believed to be BDE-209 metabolites, were found in the exposed fish. This study was repeated in rainbow trout by Stapleton et al. (2006). BDE-202 was identified as the dominant breakdown congener. The uptake of BDE-209 into the fish was determined to be 3.2% based on the body burden of the hepta- through deca-BDEs. This evidence shows that determining the bioavailability of Deca-BDE is more complex than simply determining its body burden, as in some organisms degradation products such as partially debrominated PBDE congeners may predominate.

In a review of the developmental neurotoxicity of PBDEs (Costa and Giordano, 2007) discussed several dose response relationships including: spontaneous behavior (hyperactivity and decreased habituation) and disruption in learning and memory. It is believed that these effects are in large part due to disruption of the thyroxine system; specifically, by reducing circulating thyroxine (T4). Thyroxine is involved in metabolism and neural development. There are substantial structural similarities between T4 and PBDEs. Both share the same basic diphenyl ether structure. Both also contain halogen substitutions; iodines in the case of T4 and bromines in PBDEs. Bromine is more closely related in the periodic table to iodine than the chlorines present on, for example, PCBs. T4 is converted to T3 in the body where thyroid hormone activity is required. by removal of a meta-substituted iodine atom from the phenyl ring. (Figure I.5).

Figure I.5: Structure of Thyroid Hormones



(http://www.drharper.ca/new_page_12.htm)

Endocrine receptor sites may exhibit cross reactivity.with chemicals that bear structurally similar to the intended hormone. It is possible to suppress (antagonist) or trigger (agonist) endocrine responses with synthetic chemicals. Antagonist molecules have the ability to block hormones or agonist molecules from receptor sites. This results in an inhibition of chemical signals. Agonist molecules bind with receptor sites and activate bio-chemical pathways. Molecular affinity constants measure the ratio of two chemicals bound together in solution versus the free forms of those chemicals in solution. These constants provide a quantitative tool for comparison when considering a variety of endocrine disrupting compounds (EDCs). Pathways other than the traditions agonist/antagonist actions exist. Protein transport can be equally important in delivering hormones and agonists/antagonists to active sites. The organism is not a static system and metabolic transformation pathways must also be considered. A more holistic view of the organism must also account for the probable mechanisms of toxic action for the given compound. A variety of studies have been conducted over the years to evaluate the toxicity of PBDEs. Acute toxicity of PBDEs appears low. Toxicity is generally thought

to be greatest for congeners with four to six bromines and to be mediated by bioavailability. PBDEs have been shown to disrupt endocrine function, resulting in neurodevelopmental abnormalities, delayed puberty and reproductive issues (Zhou et al., 2003; Stoker et al., 2005; Costa and Giordano 2007). The nature of PBDE toxicity is strikingly similar to that of the structurally-related PBBs and non-coplanar PCBs. Dose-dependent relationships between contaminants and toxic effects are paramount within toxicology and risk assessment science. Toxic compounds that bioaccumulate are especially important as this increases exposure. The summary of toxicology studies below focuses on observed dose-response relationships between PBDEs and effects.

In 2001, Zhou et al. examined the impacts of oral administration of Penta-BDE, Octa-BDE, and Deca-BDE commercial formulations on young female rats. Thyroid hormone levels and hepatic enzyme activities were measured following four days of exposure at a variety of dosages ranging from 0.3 mg/kg/day to 300 mg/kg/day. Thyroxine (T4) levels were depressed in the rats treated with Penta-BDE (DE-71) and Octa-BDE (DE-79). Significant decreases in T4 were observed with dosages of 10 through 300 mg/kg/day. Thyroxine decreased by 80% in the treatment group with the highest Penta-BDE dose. Thyroxine levels were 70% lower than control in the group treated with the highest dose of Octa-BDE. Another important finding of this study was the dose dependent induction of hepatic phase I (CYP1A) and II (UGPDT-glucuronosyltransferase is a type of membrane protein residing within cells in the smooth endoplasmic reticulum) enzymes in the Penta-BDE and Octa-BDE treatment groups. Significant increases of hepatic enzymes were observed beginning with 30 mg/kg/day

dosage of both the Penta-BDE and the Octa-BDE. Deca-BDE did not cause any significant differences in any of the parameters measured in this study.

In a pubertal assay designed as a Tier 1 test for EDCs, male Wistar rats were orally exposed for three days to a commercial Penta-BDE mixture (Stoker et al., 2005). A significant dose-dependent decrease was found in the weights of the ventral prostate (beginning at 30 mg/kg), seminal vesicle (beginning at 60 mg/kg), and Cowper's gland (beginning at 120 mg/kg). This study also showed a delay in the onset of puberty in rat pups orally dosed with the Penta-BDE mixture at 60 and 120 mg/kg.

A group of researchers from Sweden headed by Henrik Viberg, have conducted numerous studies evaluating the impact of BDE-209 on mammalian brain development. Viberg et al. (2003) reported that BDE-209 can pass through the blood brain barrier in mice. Furthermore, the staggering of the doses throughout the neonatal period allowed for the identification of the most sensitive development stage for BDE-209 exposure, i.e. day 3 following birth for the mice. Neurobehavioral effects were only detected in the mice treated on this day. Additionally, the effects observed in these individuals became more extreme as the mice aged.

This study design was then repeated in rats (Viberg et al., 2005). Abnormal behavior was observed in young rats two months after being orally dosed with BDE-99. This study also showed a dose-dependent decrease in muscarinic cholinergic receptors in the same rats. The response was statistically significant beginning at the 16 mg/kg body weight dose. These findings mimic earlier work exposing mice to PCBs (Eriksson et al., 1991). This study (Viberg et al., 2005) showed that BDE-99 can create persistent

neurotoxic effects in mice if exposure occurs during a critical phase of neonatal development.

Another study was run, mimicking the methods of the previous two, to determine what the impact of BDE-209 would be on developing mammalian brains (Viberg et al., 2007) The results of this study supported the earlier findings in mice. In addition, this study included a nicotine-induced behavior test. The key finding here was that nicotine exposure led to hyperactivity in control and low dose BDE209 treated mice. However, the animals exposed to the high dose of BDE-209 (21 mg/kg bw) showed hypoactivity following nicotine exposure. The implications of this finding are that the cholinergic system is a target for BDE-209-induced developmental neurotoxicity.

The same research group ran another study examining the impact of BDE-209 on mammalian brain development (Viberg et al, 2008). This time the brains of the animals were dissected into various components, including the cortex, the hippocampus, and the whole brain. The concentrations of three proteins were measured in the tissues seven days after exposure. There were significant differences between the mean concentrations of all three proteins in various parts of the brain. The three proteins investigated regulate different neuronal processes associated with survival, growth, and synaptogenesis. Building upon this work, a similar study was published in 2009 which investigated the impacts of BDE-209 on two additional neural proteins. Significant differences in protein levels were not detected for the two proteins examined (Viberg et al., 2009).

Viberg et al's 2008 study was criticized by flame retardant industry representatives (Hardy and Stedeford, 2008). Hardy and Stedeford criticized Viberg's use of individual pups from the same litter as an experimental unit in the study discussed

above. Hardy cites EPA methodologies that require toxicity studies control for the "litter effect" in mammalian studies. Hardy made a similar criticism of another BDE-209 rat exposure study by Van der Ven et al. (2008a). Van der Ven et al.'s rebuttal is relevant to the criticism of the Viberg et al. (2008b) study. Acknowledgement is made that there is a difference of opinion in the scientific community about the statistical methods that must be used when dosing pups in the neonatal period. Van der Ven goes on to note that the litter effect is of paramount importance when the dam is exposed and the pups are evaluated for effects. However, Viberg et al's studies did not expose the dam. Instead male pups were exposed after birth in the neonatal period. In addition, Viberg et al maintained statistical power by using a minimum of three litters for each treatment group. So the individual pups were used as experimental units, but they do not ALL come from the same litter.

Van der Ven et al. (2008) conducted a 28 oral dose toxicity study of Deca-BDE in Wistar rats. This study replicated the gavage and emulsion method designed by Morck et al., (2003). The most sensitive effect in males was an increase in weight of the seminal vesicle, beginning at 0.2 mg/kg bw/day, and increased expression of CYP1A and CYP2B (0.5-0.7 mg/kg bw/day). In females, decreased activity of P450c17 (CYP17), a key enzyme in the androgen synthesis pathway, was observed. The authors postulated that this result may indicate that Deca-BDE poses a reproductive health hazard.

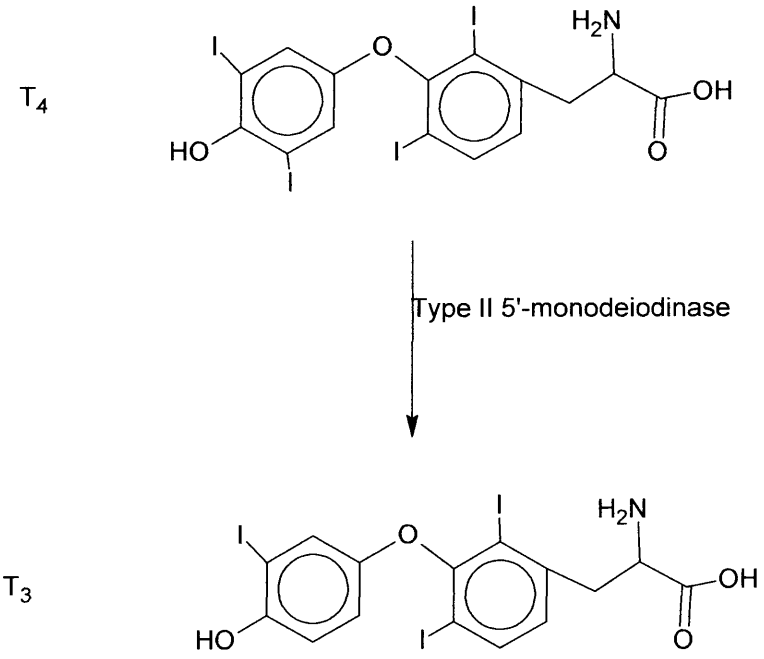
I.4: Metabolism of BDE-209

In mammals BDE-209 is metabolized through an oxidative pathway to form a variety of hydroxylated PBDEs (EPA, 2008; Morck et al., 2003; Sandholm et al., 2003).

It is likely that this is mediated by an induced CYP1A reaction triggered by interaction of BDE-209 with the Arylhydrocarbon receptor (Ahr). The other possible mechanism for metabolism of BDE-209 is the reductive debromination of the molecule. For this to occur one of the bromine atoms is removed and replaced with a hydrogen atom. The products of this pathway would be lower brominated PBDE congeners; which are known to be more toxic than BDE-209. These would also be more strongly retained than hydroxylated PBDEs It appears that the reductive pathway occurs in fish (Stapleton et al., 2006). In adult birds, this may also be the case (Van den Steen et al., 2007).

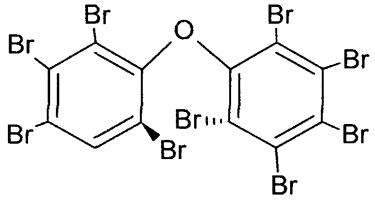
Activation of thyroxine (T4→T3) requires the removal of an iodine atom from the 5' position of a diphenyl ether structure (see Figure I.6). Chicken embryos show 5'-monodeiodinase activity (5'-MA) in both the liver and the brain. Valverde et al. (1993) reported that deiodination activity ceases in the brain around Day 15 of embryonic development. Valverde et al. made the observation that 5'-MA peaks at day 18-20 in liver of chicken embryos.

Figure I.6: Activation of T4→T3

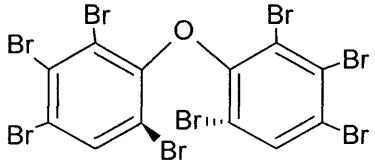


In BDE-209, the diphenyl ether structure is fully brominated. Assuming dehalogenation of BDE-209 is facilitated by 5'-MA, logical degradates would be nona- and octa- PBDE congeners, lacking bromines at the position 5 carbons. These would bde BDE-207 and BDE-197. See figure I.7. The argument can also be made that other deiodinases may be present with the ability to remove halogen atoms from all meta positions. The thought is that the active site of the enzyme might not be specific only to position 5, rather only specific to the meta postion of the ether linkage. This would create preferential formation of nona- and octa-BDEs with unsubstituted 3 and 5 position carbons. Van der Ven et al. (2008) reported production of BDE-207 in the livers of Wistar rats exposed to BDE209. The ratio of BDE207 to BDE209 was used to track biotransformation over their 28 study period.

Figure I.7: BDE-207 and BDE-197



BDE-207



BDE-197

Debromination of BDE-209 in adult starlings was recently investigated by Van den Steen et al. (2007), using silastic implants to deliver BDE-209 into the blood stream of starlings. At the conclusion of that study the BDE-209 concentrations in the tissues of the control birds were below the limit of quantitation of 5.6 ng/g lw (muscle) and 2.9 ng/g

lw (liver). In the exposed birds the muscle tissue concentrations of BDE-209 were about twice that of the liver concentrations (430 ng/g lw and 237 ng/g lw respectively.) They postulated that the liver possessed greater metabolic activity than muscle, reducing associated BDE209 burdens. This was supported by the observation that the relative concentrations of the nona-BDEs were greater in the liver than the muscle tissue. This study showed that, while adult birds are capable of debrominating BDE-209, the chemical still accumulated in avian tissues. Eggs have been observed to contain both BDE-209 and apparent debromination products.

I.5: Egg Exposure

Egg injection bioassays have been developed and tested since the 1960s. A variety of methods exist to deliver compounds to a developing avian embryo. Many researchers have explored exposing the outer egg shell to petroleum products and insecticides. There are two main distinctions between egg injection methodologies. Namely, those that target the air cell of the embryo (i.e., the space between the eggshell and the outer membrane of the embryo) and those that target the yolk.

Some of the earliest yolk injection studies were designed to evaluate the efficacy of this test in determining comparative toxicity of different compounds on avian growth and development (Walker 1967, McLaughlin et al. 1963). In this sense, the findings of Walker (1967) findings were definitive on the matter. "Chemicals and mixtures of various types injected into the yolks of fertile eggs moved and reacted in different ways, depending upon their densities and individual properties". Clearly, results of tests with

different modes of toxicant delivery, and therefore different organismal exposures, should be treated with caution.

Walker's findings remain crucial in understanding the dynamics of yolk injections. Further investigations have been conducted to compare the impact of a given toxicant when injected into either the yolk or the air cell of a chicken embryo. Henshel et al., (1997) published a study comparing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity in developing chicken embryos by these two injection methods. Findings showed the embryo was more sensitive to TCDD when injected into the yolk (LD50 = 122 pg/egg) than the aircell (LD50 = 297 pg/egg). Henshel's findings are the opposite of Gebhardt and van Logten (1968). Gebhardt and van Logten tested the comparative toxicity of a dithiocarbamate in yolk versus air cell injections to the chicken embryo. They reported that for these toxicants, the embryos were more sensitive to the thiram injected into the aircell (LD50 = 1.1 μg), rather than into the yolk (LD50 = 18 μg). In order to understand these inconsistencies, Walker's findings must be revisited. Henshel provides a compelling discussion of these results within the context of Walker's findings.

Transfer of contaminants from hens to eggs occurs as a continuous process as the egg is created within the bird. Over time the mother lays down nutrient layers in the yolk for the developing embryo to consume throughout the development process. There is an order and structure to the creation of the ovum. Walker (1967) documented the behavior of a variety of chemicals when injected into the yolk of the developing chicken egg. Using various dyes, Walker (1967) tracked the movement and disposition of various chemicals in chicken yolks. Oils were found to not mix well at all with the yolks, rather they rose to the top of the yolk as a "single bubble". Walker reported low mortality for

vegetable oil yolk injections but high mortality for other compounds that behaved similarly; rising as a single bubble when injected to the yolk. This is likely due to the close proximity of the "bubble" with the blastodisc, which also rises in the yolk and rotates as the eggs are turned throughout incubation

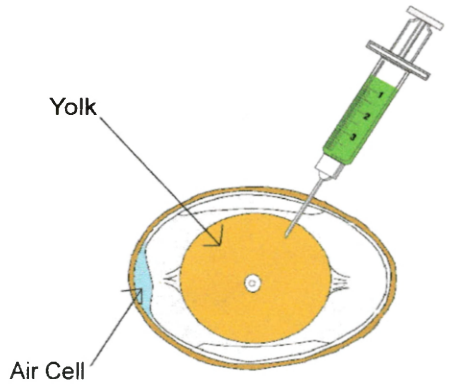
Henshel's reasoning illustrates many of the facets of the yolk injection exposure approach. The nature of the carrier may define the exposure, as much as, if not more than the nature of the toxicant itself. Henshel calls for the evaluation of results within the context of the injection site and the impact it may play on both physiological and physical processes occurring within the egg. Figure I.8 below is Henshel's Appendix. It presents a matrix of the advantages and disadvantages inherent in each of the two methods.

Figure I.8: Appendix from Henshel et. al. 1997 p 732

	Air cell	**Yolk**
Benefits	1. Able to inject throughout incubation.	1. Able to inject a larger amount of chemical without causing nonspecific embryo mortality.
	2. Applied directly onto embryo. This is especially useful if the chemical is readily taken up by the embryo.	2. Oil or emulsion-injected material stays near the top of the yolk, readily accessible to the embryo.
	3. Vehicle is not as critical as for the yolk, although it may define the maximum injection volume	3. Material taken up by the embryo by "natural" processes during yolk absorption, and distributed with all nutrients through the embryo
Drawbacks	1. Might inject into albumin, and lose early embryo exposure. (Albumin is used by the embryo in the latter half of the incubation period.) This will also cause a decreased apparent toxicity for chemicals that also affect early embryos.	1. Not as good for later embryo injections, as one is more likely to damage the embryos or the vitelline vasculature and cause potential hemorrhage or abnormal growth.
	2. If very small quantity is injected, it is possible that the embryos may not be exposed to the substance immediately because a very small amount of substance could move around the embryo rather than across the embryo.	2. Oil or emulsion tends to be more concentrated and is not diluted by entire yolk, so embryo is exposed to higher concentrations earlier in incubations than if chemical mixed well with yolk.
	3. Maximum recommended total injection volumes to avoid vehicle-related embryo mortality: 50 µl for oil-based; 100 µl for water-based.	3. Some diluents or solvents interact with yolk (e.g. propylene glycol and formaldehyde), and therefore are not accessible to the embryo, producing falsely high medial lethal doses (LD50s) and median effective doses (ED50s).

There are several reasons for selecting the yolk over the air-cell injection method with regards to Deca-BDE: 1) Carriers commonly used in the air-cell injections of the Penta- and Octa-BDEs do not solubilize Deca-BDE well 2) While the air-cell is within the eggshell it is separated from the embryo by a membrane. This means that materials injected into the air cell must first pass through this before contacting the embryo. By injecting material directly into the yolk of the egg, the food source for the embryo, the material is immediately available (See Figure I.9).

Figure I.9: Egg Yolk Injection



Maternal transfer of nutrients into the egg is done in a structured manner; nutrients are laid down in layers. Obviously, it is impossible to replicate the exact internal structure and distribution of a contaminant as transferred from hen to egg, without conducting a feeding study of the hens. Yolk-injection was chosen for this study because it allowed the preservation of a closed system within the egg. However, the author desired to have the distribution of yolk-injected BDE-209 similar to what occurs from maternal transfer. The findings of Walker (1967) are definitive on the behavior of

various chemicals when injected into the yolk-sac of chicken eggs. Walker determined that injection of water soluble compounds mixed well with yolk, while oils tended to move "en masse" with no sign of mixing. An emulsion formulation was chosen because of the high water content, 78% (w/w) for the emulsion used in these studies. An emulsion by definition contains oils. Therefore, it is likely that the yolk-injected material was not dispersed evenly throughout the entire yolk. It is likely that the emulsion spread somewhat from the original "bubble". It is also likely, that some of the original "bubble" remained cohesive. In this case, the embryos would have experienced greater exposure as the bubble rotated with the embryo inside the eggs throughout incubation.

I.6: Purpose

The purpose of this study was to examine the biological partitioning of BDE-209, the major congener of Deca-BDE, in an avian embryo. In the US, the use of Deca-BDE is currently unregulated at the federal level, although restrictions on some specific applications are in place in several states[4]. Restrictions are also under consideration in Canada and Deca-BDE has been banned in new products in the EU. It remains in extensive use around the globe as a flame retardant additive in numerous consumer products. The EPA TRI indicates substantial Deca-BDE releases directly to the environment and through wastewater treatment plants. Releases directly from in-use products also occur, as evidenced by its presence at mg/kg levels in indoor dust. Numerous studies have detected BDE-209 in the environment, and it is often the most abundant congener in abiotic media such as sewage sludge, soil and airborne particulates.

[4] (http://www.bsef.com/regulation/north-america/deca-bde-3/)

However, until recently, it was not reported in biota. Rather, the constituent congeners of Penta-BDE (BDE-47, 99, 100, 154 and 154) were typically reported, especially in aquatic organisms and on occasion at concentrations in excess of 10 mg/kg (lw). Previous studies have shown that BDE-209 is environmentally persistent and widely distributed. Based on its large molecular size, strong sorption to particles and low water and lipid solubility it was originally believed to pose a minimal environmental hazard. This supposition was supported by studies of aquatic species, which showed almost no bioaccumulation. However, substantial burdens of BDE-209 have more recently been observed in some terrestrial birds. In addition, nona- and octa-brominated congeners, thought to be derived from BDE-209, have also been reported in raptors and their eggs. Similar congeners have been observed in fish near large BDE-209 point sources (La Guardia et al, 2007), as well in laboratory feeding studies (Stapleton et al, 2004). These indicate that a fuller understanding of routes of exposure is critical to evaluate the true toxicological ramifications of continued Deca-BDE use. . Much lower BDE-209 levels have been observed in terrestrial mammals than in raptors. Bird eggs are a widely used environmental matrix to monitor contaminant levels. The toxicology literature indicates that BDE-209 is rapidly excreted by some mammals (Norris et al. 1975 and Morck et al. 2003). Van den Steen et al. (2007) was the first to demonstrate debromination of BDE-209 in birds in the course of an in-laboratory exposure. There has been limited work to date examining the toxicological impacts of BDE-209 on developing avian embryos. This is especially relevant as BDE-209 and less brominated congeners, believed to be BDE-209 degradates, are known to accumulate in terrestrial birds and their eggs.

It is uncertain whether these metabolic byproducts are produced in-ovo or are pre-existing in the resources transferred from the hen to the egg. The lower brominated PBDEs have typically been reported to have higher toxicities and bioaccumulative potentials than BDE-209. Therefore, if BDE-209 is debrominated in the mother hen and then transferred to the eggs, there is the potential for enhanced toxicity at the beginning of embryonic development. If BDE-209 is degraded in the embryo to more toxic metabolites then toxicity may increase throughout embryonic development. This study is designed to address the latter question.

Tissue distribution is important to consider as well, as chemicals may interfere with the function of specific organs or biochemical pathways. For example, numerous studies (Viberg et al. 2003, 2005, 2007, and 2008 amongst others) have shown that BDE-209 is developmentally neurotoxic to mammals during the brain growth spurt on day 3 of life. This study will assess the disposition and acute toxicity of BDE-209, and possible partial debromination products, in the developing embryo and various critical organs.

Chickens (*Gallus gallus*) show high sensitivity to toxicants that bind with the Ahr, and relatively low sensitivity to toxicants which cause egg-shell thinning such as DDT. The chicken was chosen as an avian model for this study as it is: readily available, easy to maintain in the laboratory and is a major food source for humans. Substantial levels of BDE-209 and assumed degradates have been reported in some terrestrial avian species, including domestic chickens near point sources of Deca-BDE such as electronic recycling sites, as well as in feral raptors (Liang et al. 2009). The current prevailing hypothesis is that BDE-209 (the major component of the Deca-BDE commercial product) is more prevalent in terrestrial than aquatic organisms. Concentrations of BDE-209 in some

terrestrial birds (130-2000 ng/g lipid) have been observed to exceed by over an order of magnitude those reported in mammals ($\leq$ 41 μg/kg lipid) (Lindberg et al., 2004; Chen et al., 2007; Christensen et al., 2005). This study will examine the distribution of BDE-209 following injection into the yolk-sac of chicken embryos. The egg yolk injection methodology is also expected to maximize the absorbed concentration of BDE-209, allowing for an increased likelihood of detecting it and any related debromination products.

MATERIALS AND METHODS

M.1: Dosing Formulation

An emulsified formulation of BDE-209 was developed for yolk injection into chicken eggs. The basis for the formulation came from a study (Morck et al., 2003) run in mammals where the exposure vehicle was designed to better disperse the dose of this extremely hydrophobic PBDE and mimic stomach contents. The emulsification agents used were Lμtrol® micro 127 MP and Phospholipone® 90G from BASF Corp. (Florham, NJ) and Lipoid LLC (Newark, NJ), respectively. Decabromodiphenyl ether (BDE-209) was ordered from Wellington Labs (Guelph, Ontario, Canada). Preliminary work was done with commercial preparations of Deca-BDE. However, a highly purified (>98%) BDE-209 standard was required for this study as a major interest was the potential for biotransformation to less-brominated congeners. The BDE-209 was first dissolved in a 2:1 v/v toluene and diethyl ether solvent mixture. This required the use of a probe sonicator (type) for 30 minutes. The BDE-209 solution (volume) was then added to an emulsion vehicle base composed of a mixture of Lμtrol® micro 127 MP and Phospholipone® 90G in a ratio of 34:16 in water. The remaining water was then added to the solution drop-wise while mixing. The final step was to evaporate off the solvents using a purified N_2 stream. A total of five treatments were developed; four different

doses of BDE-209 and an emulsion vehicle. The nominal concentrations of the doses were 80 μg/egg, 40 μg/egg, 20 μg/egg, and 5 μg/egg. The highest dose (80 μg/egg) was administered in 100 μl injections. The rest of the doses were administered in 50 μl injections. Therefore the high dose emulsion with a concentration of 800 μg/ml of emulsion, was the same emulsion used in the 80 μg/egg and 40 μg/egg treatment groups. This is discussed in more detail below.

M.2: Dose Verification

Nominal concentrations of the BDE-209 emulsions were verified analytically following the methods of LaGuardia, 2008. The extraction method was designed by the author with assistance from Mr. LaGuardia. The high, medium, low and vehicle treatment emulsions were subjected to liquid/liquid extraction using dichloromethane (DCM) and quantification by gas chromatography and mass spectrometry (GC/MS) in the electron capture negative ionization (ECNI) mode. Briefly, one ml of each emulsion was first diluted with 100 ml of DI H_2O before being subjected to three 5 ml extractions with DCM in a separatory funnel. Each wash with DCM was shaken for five minutes before allowing the layers to separate and collection of the organic layer. The organic layers were collected in an appropriate volumetric flask for dilution into the range of the calibration curve from 50 ng/ml to 5000 ng/ml. The extract of 1 ml of the high dose emulsion was diluted with hexane into a 500 ml volumetric flask. An aliquot of that dilution was then analyzed by GC/MS. The ions of interest 79 and 81 (*m/z*) were monitored throughout the whole run and used to integrate and quantify BDE-209. The ion 486 was used to confirm BDE-209. The expected concentration from this 500-fold

dilution was 1600 ng BDE-209 per ml. The extract of 1 ml of the medium dose emulsion was diluted with hexane in a 250 ml volumetric flask. An aliquot of that dilution was then analyzed by GC/MS. The expected concentration from this 250-fold was also 1600 ng BDE-209 per ml. The extract of 1 ml of the low dose emulsion was diluted with hexane into a 100 ml volumetric flask. An aliquot of that dilution was then analyzed by GC/MS. The expected concentration from this 100-fold dilution was 983 ng BDE-209 per ml.

The samples were then spiked with 300 ng of decachlorodiphenyl ether (DCDE; CAS# 3170-30-2) as an internal standard and analyzed by GC/MS in the NCI mode. Congener peak areas generated were compared to that of the internal standard added to the extract. Response factors were determined using authentic PBDE standards from AccuStandard (New Haven, CT, USA) and Cambridge Isotope Laboratories (Andover, MA, USA).

Instrumental analysis followed that was described previously by LaGuardia et al. (2006) and Chen et al. (2008). Final extracts were analyzed by GC (6890N, Agilent Tech., Palo Alto, CA) with MS detection (JMS-GC Mate II, JEOL, Peabody, MA). Ion fragments were produced in the ECNI mode using methane (99.99%) as the reagent gas. Injections (1 μl) were made into the splitless injector equipped with a 1 mm i.d. glass liner. A 15 m DB-5HT column (J&W Scientific, Agilent Technologies, 0.25 mm ID, 0.1 μm stationary phase thickness) was installed in the GC. Helium was used as the GC carrier gas at an initial pressure of 50 psi. The injection technique used, "pressure pulse split-splitless", shows minimal thermal degradation of BDE-209 (LaGuardia, 2008). Initial column temperature was 90°C and held for 4 minutes. Temperature was then

increased to 150°C at 30°C/min, then 10°C/min to 300°C and held for 7 minutes. The column temperature was then increased to 350°C for 5 minutes as a bake out procedure.

M.3: Eggs and incubations

All egg incubation methods followed those from McKernan et al. (2009). Dr. McKernan was instrumental in the completion of this work, and all experiments at Patuxent (PWRC) were run under her scrutiny. Preliminary work with the emulsion vehicle in chicken embryos was conducted at VIMS (Gloucester Point, VA, USA). All of the procedures involving animals were approved by the Institutional Care and Use Committees of the College of William and Mary and PWRC. CBT Farms (Chestertown, MD, USA) supplied the fertile white leghorn chicken (*Gallus gallus*) eggs used in studies employing viable embryos. All of the eggs were washed in a 40°C 1% Betadine® solution (Purdue, Wilson, NC, USA) upon arrival. Each egg was then weighed and labeled with a number 2 graphite pencil. The eggs were then stored in a cooler at 13°C for up to 3 d. Eggs were allowed to equilibrate to room temperature before placement into incubators. Eggs were artificially incubated (Kuhl Incubator Company, Flemington, NJ) in trays that rotated eggs horizontally (180 degrees per hour). Incubators were set at 37.6°C, as is recommended for chicken eggs (Henshel et al., 1997). The relative humidity within the incubator was originally set at 40%. Egg weights were monitored every 3 to 4 days throughout incubation. The relative humidity was adjusted so the mean egg weight loss at the end of incubation was 14 to 16%. Eggs were candled at the time of weighing to confirm viability. Any unfertilized or dead eggs were removed.

M.4: Dose Administration

These methods were adapted from Quinn et al. (2008) for species differences between Japanese quail and chickens. Prior to injection, the blunt end of each egg was cleaned with an alcohol swab and was penetrated using a drill (Dremel, Racine, WI USA). Two different injection volumes were used in this study; 50 μL and 100 μL. This is why the various doses were all delivered on a per egg basis. The volume of the yolk-injected dose and control treatments were kept constant in all trials. The doses were administered to the developing embryos by injection into the yolk-sac using a Hamilton syringe with a 22 gauge needle. To determine the proper depth to insert the needle, eggs were measured lengthwise. The average length was then divided by 2 to determine the center of the egg. A pipette tip was then measured and placed over the tip of the needle to act as a stop. This allowed the injection to target the center of the egg every time. Preliminary work was conducted with water and dye to confirm the location of the injection within the embryo. Separate syringes and needles were used for the vehicle and treatment groups to prevent contamination. The hole in the egg was then sealed with ethylene vinyl acetate adhesive using a hot glue gun. All eggs were kept out of the incubator for an equivalent 30 minutes. Eggs were then placed in trays and positioned horizontally into the incubator.

M.5: Monitoring Survival and Sample Collections

The methods followed have been described by McKernan et. al.(2009), except for a modest change in the sample collection timing. Embryo survival was monitored at 3 to 4 d intervals by candling or with a viability detection instrument (Buddy, Vetronic,

Torquay, UK). The Buddy instrument detects electrical impulses from both the beating heart of the embryo and any motor movements occurring within the egg. Embryos that died during development were removed from the incubator and stored in the freezer for chemical analysis. The endpoint of this study was the 20th day of incubation, as this is the last day before pipping and followed the peak in liver enzymatic activity. As the goal was to evaluate in ovo fate of the BDE-209, it was imperative that the chicks were sampled before they left the closed system of the egg. On Day 19 or 20 the chicks were removed from the eggshells, weighed, and examined for evidence of edema and teratogenicity (e.g. eye, foot or bill deformities). The birds were then sacrificed by decapitation. The liver, heart, brain and remaining yolk-sac were promptly removed and weighed.

M.6: Trial 1: Evaluation of emulsion vehicle-induced embryonic mortality, VIMS, December 2007

A total of 20 eggs from CBT Farms in Chestertown MD were received on 12/4/2007. The eggs were candled. One egg was cracked and therefore discarded. The eggs were then placed in an incubator and maintained at 37°C. Humidity levels were not monitored, but water was regularly added every few days to the lower tray of the incubator. At Day 4 all the eggs were candled again. Six of the 19 eggs were not viable at Day 4 and were discarded. The remaining 13 eggs were randomly assigned to one of three treatment groups; uninjected (n = 4), deionized water (DI) water injected (n = 4), or emulsion vehicle injected (n = 5). Assignment was done randomly by rolling a die, two numbers were selected for each treatment group. The emulsion injection volume used

was 100 µL. Incubation was continued through Day 19. The remaining embryos were then removed from their shells, sacrificed and necropsies conducted. Tissue weights were recorded for the yolk, liver, and brain.

M.7: Trial 2: Evaluation of emulsion-induced embryonic mortality, VIMS, February 2008

A total of 47 eggs from CBT Farms (Chestertown MD) were incubated beginning on 2/15/2008. The eggs were then placed in an incubator and incubated at 37°C. Humidity levels were not monitored, but water was regularly added every few days to the lower tray of the incubator. At Day 4 all the eggs were candled again. Two of the 47 eggs were not viable at Day 4 and were discarded. The remaining 45 eggs were randomly assigned to one of three treatment groups; uninjected (n = 15), sham-injected (n = 15), or vehicle injected (n = 15). The "sham" injections mimicked the emulsion vehicle injections, in that the needle was pushed into the yolk. The only difference between the "sham" and vehicle emulsions is that no liquid was delivered to the yolk in the sham injections. Treatment assignment was done by tossing a die. The injection volume used was 100 µL. At Day 19, the embryos were removed from their shells, sacrificed and tissue weights determined.

M.8: Trial 3: Testing the effects of a single BDE-209 dose on embryonic mortality, Patuxent Wildlife Research Center, June 2008

Beginning on June 11, 2008 a trial of 198 eggs was run. Initiation and termination of the trial was staggered over a three day period. This resulted in three

replicates of each treatment group. On Day 4 of incubation all the eggs were randomly assigned to one of three treatment groups. The treatment groups in this trial were uninjected (n = 26), vehicle injected (n = 80), and BDE-209 injected at 80 μg/egg dose (n = 79.) The injection volume in this trial was 100 μL. Mortality and moisture weight loss were monitored throughout the incubation period. At Day 19, the embryos were removed from their shells, sacrificed and necropsied.

M.9: Trial 4: Testing the effect of multiple doses of BDE-209 on embryonic mortality, Patuxent Wildlife Research Center, July 2008

Multiple doses of BDE-209 were injected into chicken yolks. The injection volume for this trial was 50 μL. The high dose was 40 μg/egg or 800 μg/ml of emulsion (n = 47). The middle dose was 20 μg/egg or 400 ug/ml of emulsion (n = 49). The low dose was 5 μg/ egg or 100 μg/ml of emulsion (n = 43). A total of 30 eggs were also run as uninjected controls. Again the initiation and termination of this trial was staggered over a three day period. This resulted in three replicates of all five treatments. Mortality and moisture weight loss were monitored throughout the incubation period.

On day 19 the embryos were sacrificed by decapitation. The birds in the middle dose and vehicle treatment groups were necropsied. Five compartments were examined; liver, heart, brain, yolk, and remaining carcass. These compartments were all weighed prior to being collected in solvent-rinsed glass jars for further analysis. The tissues and the yolks from the individual birds were pooled together. Four birds were pooled per composite sample. So, one set of the five tissue samples contained the tissue from four individual birds.

M.10: Experiment 2: Absorption and distribution of yolk-sac administered BDE-209

For the chemical distribution aspect of the study, samples were collected from only the Middle dose (20μg/egg) and Vehicle treatment groups from Trial 4. The Middle dose treatment group was selected because of the sample size that survived through to the end of incubation. Due to the small weight of the tissues collected and the expected low attendant amounts of analyte present, the samples were pooled. Birds within a treatment group (n = 32 per group) were selected at random for pooling. Four birds were selected from each treatment for each pool. In an effort to control the total number of samples and provide statistical meaning, the number of birds collected from each group was predetermined to be 32. Each pool of four birds resulted in five pooled samples total, including: livers, hearts, brains, pooled yolks, and carcasses. The total sample size for each compartment within each treatment was eight. The birds were necropsied sequentially and the organs were collected in solvent rinsed glass jars.

All tissue samples were subjected to chemical analysis as described by LaGuardia et al. (2007) and Hale et al. (2001). The first step was freeze-drying for a period of 48 hours (Dura Top, FTS Systems, Stony Ridge NY). Tissue samples and sodium sulfate blanks were spiked with a surrogate standard consisting of 1000 ng each of PCB-204 and BDE-166. Dried samples were extracted using enhanced solvent extraction (Dionex ASE 200, Sunnyvale CA). Two 5-min. extractions were performed per sample with DCM at 100°C and 1000 psi. Gravimetric analysis of a 10% subsample of the extract was conducted to determine DCM soluble lipids. Next, size exclusion chromatography

(SEC) was performed to remove the large molecular weight biogenic lipids from the analytes of interest (Envirosep-ABC, 350 x 21.1 mm column; Phenomenex, Torrance, CA). The final purification step was conducted using a 2 g, silica gel, solid phase extraction column (Isolute, International Sorbent Technology, UK). The first fraction was eluted using 3 ml of hexane and discarded. The second fraction (S2), containing the halogenated compounds of interest, was eluted using 10 ml of a 90:10 v/v hexane/DCM solvent mixture and retained. A third fraction (S3) composed of an additional 5 ml of the 90:10 hexane/DCM mixture was also eluted and collected. The columns were then eluted with 9 ml of acetone (S4) to remove any remaining potential analytes of interest. The S2 fractions were reduced to near dryness under purified nitrogen and exchanged to hexane. These were then spiked with 300 ng of the internal standard DCDE. Instrumental analysis followed that was described previously for the dose verification portion of this study.

M.11: Data Analysis and Mass Balance

A major goal of this study was to determine the fate and behavior of BDE-209 in the developing chicken embryo. To best accomplish this, a mass balance approach was applied. The mean concentration values of the different tissue samples were multiplied by the total tissue weights. The sum of these values should equal the original treatment dose. If the mass measured is less than the amount dosed then it is likely that not all of the BDE-209 made it through the analytical process, there was biotransformation of the BDE-209 into other chemicals or errors exist in the measurement of dosage of tissue residues. Use of pooled tissue samples will also result in some error.

It should be noted that the high lipid content of the yolk and carcass tissues may also create matrix interferences in the MS detector. The extraction and cleanup methods discussed below were optimized to minimize any matrix induced signal interference.

M.12: QA/QC and Method Development

The samples collected from trial four were chemically analyzed for BDE-209 and potential PBDE-debromination products. Originally all 80 samples (5 tissues with 8 replicates in two treatments groups) were freeze dried in preparation for organic extraction. Preliminary work was done with the yolk and carcass samples from the vehicle treatment group, as there was ample sample available from these two tissue compartments. The method originally tested is described by LaGuardia et al. (2006) and Hale et al. (2001) and is used routinely on fish samples to quantify PBDEs and other organic contaminants. Unfortunately, employing this method with the chicken egg matrix resulted in GC/MS interference issues. In spike recovery studies, the BDE-209 signal in the yolk and carcass matrices was amplified 1.5 to 2 times that of the standards. This was attributed to excess biogenic material in the final purified extract. Therefore, modifications to eliminate more of this material from the samples were required.

The solid-phase extraction (SPE) step was the final step for the elimination of co-extracted biogenics. Hence, various column elution options were examined. A weaker solvent regime would likely elute off less material. Therefore, initially the utility of 100% hexane was examined. BDE-209 was eluted, but required a high volume (15 ml) of solvent. Next, a solvent regime of 10% DCM and 90% hexane was next tested. Three replicates were run. The columns were prepped with 5 ml of 100% hexane. The SPE

columns were spiked with 1000 ng BDE-209, 480 ng PCB-204, and 480 ng BDE-166. The first fraction was eluted with 3 ml of hexane and discarded. The second (S2) fraction consisted of 8 ml of 10% DCM 90% hexane. This was collected, reduced to < 1ml and spiked with 300 ng DCDE before GC/MS analysis. The third (S2_A) fraction consisted of an additional 3 ml of 10% DCM. The fourth and fifth fractions (S2_B and S2_C, respectively), were also 3 ml of 10% DCM. These were also reduced to <1ml and spiked with 300 ng of DCDE as an internal standard.

Based on the data from the spiked column trial discussed above, a spike recovery test was run using locally-bought eggs. A dozen large white eggs (nonviable) were purchased from a commercial grocery chain store (Food Lion, Yorktown, VA, USA). The yolks were collected from six eggs, pooled, homogenized and freeze dried. For the other six eggs the yolks and the egg whites were combined, homogenized and freeze dried. Three replicates of each matrix, as well as three blanks consisting of sodium sulfate were processed. Each sample was then spiked with 1ml of a composite standard containing 2000 ng/ml BDE -47, -99, -154, -183, and -209. as well as 820 ng/ml of PCB-204 and BDE-166. The samples then underwent extraction on the ASE (Dionex, Sunnyvale, CA, USA) followed by lipid analysis and size exclusion on the HPLC. Only 56.3% of the original sample material makes it through to the SPE step; due to mechanism for filling the injection loop. The previously described SPE column study showed that 8 ml of 10% DCM was insufficient to elute all BDE-209 from the silica gel. In the spiked matrix study, 10 ml of the 10% DCM was used to elute the S2 fraction.

Procedural blanks consisting of sodium sulfate were run with every batch of ten samples. These were spiked with the surrogate standard (BDE-166) and monitored for possible BDE-209 contamination.

M.13: Statistical Analysis

Contingency Analysis (χ^2, Chi-Squared)

The mortality data for each trial were organized into a contingency table; where the columns represent survival or death and the rows represent the various treatment groups.

Table M.1: Contingency Table, Observed Values

	Observed		
	Survive	**Dead**	**Total**
Vehicle	x	y	x+y
Deca	w	z	w+z
Total	x+w	y+z	x+y+w+z

The row totals are multiplied by the column totals and divided by the overall total to calculate expected values.

Table M.2: Contingency Table, Expected Values

	Survive	Dead	Total
Vehicle	$\frac{(x+y)*(x+w)}{x+y+w+z}$	$\frac{(x+y)*(y+z)}{x+y+w+z}$	x+y
Deca	$\frac{(w+z)*(x+w)}{x+y+w+z}$	$\frac{(w+z)*(y+z)}{x+y+w+z}$	w+z
Total	x + w	y+z	x+y+w+z

The final step is to calculate and total the differences between the observed and expected values using the following formula.

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

$$df = (r-1)*(c-1)$$

r = # of rows, c = # of columns

The null hypothesis for this test is that the treatments (rows) have no impact on the probability of mortality (columns). The alternative hypothesis is that the rows (treatments) increase the probability of one of the columns (mortality). The χ^2 value and the degrees of freedom (df) were input into Microsoft Excel to calculate the corresponding p-value from the probability density function.

Acute Toxicity ~ Calculating the LD50

The Median Lethal Dose (LD50) is a metric often used in toxicology and risk assessment. The methods used here came directly from the 1995 reference "Quantitative Methods in Aquatic Ecotoxicology" by Michael C. Newman. In order to accurately calculate the LD50 for a given compound, and method of exposure, data for multiple doses are required. Furthermore, the data set must include at least one example of a partial kill; i.e. mortality other than 0% or 100%. The first step in determining the LD50 for a given set of data is to determine the distribution of the data. This means trying to determine which model (Normal, Logistic, or Weibull) the data distribution best fits. Normally distributed data form a bell curve centered on the mean value of a given parameter in a population. Logistically distributions are similar to normal data, except

the bell shows greater kurtosis.. The Weibull distribution is more exponential in appearance.

In conducting the LD50 analysis, the data distribution plays a key role in determining the appropriateness or "best fit" of the method used. For example, if the data are normally distributed, then Probit analysis is most appropriate. If the data are logistically distributed then Logit analysis is a better tool. Finally, if the data follow a Wiebull distribution then the Gompertz model is the most appropriate. Determination of the distribution of the data and selection of the appropriate model can be done by comparing the Pearson Chi-Square value for each analysis. The Pearson Chi Square value is provided by the SAS program as a measure of "goodness of fit." The smaller the value of the Pearson Chi-Square value the better the fit of the data to the assumed distribution and corresponding model. The distribution of data points is an important consideration when fitting models It is widely accepted that the internal areas of the cumulative density function (CDF) are most accurate. This is one reason why the LD50 is used so often. The accuracy of the models is tested in the external realms of the CDFs or the tails. Hence, values for LD5 (lethal dose of 5% of the population) and LD99 (lethal dose 99% of the population) are not used as widely. These values can easily be determined once a model has been fit to the data. This is where the distribution of the data becomes important. The assumptions made at the beginning of the process have an impact on the results, especially at the extremes. This is why the Pearson Chi-Squared value is important in understanding the distribution of the data and hence the selection of the most accurate model.

The data from the last two trials were pooled to develop a dose response curve. Again, the method here follows the 1995 Newman text. These data were then input into SAS 9 and used to estimate a model of mortality that accounts for the toxicity of the emulsion itself and not the BDE-209. The data were first fit to a Probit, then a Logit and finally a Gompertz model. The model with the best fit was identified by the smallest value of the Pearson Chi-Square Value for the "Goodness of Fit" test. This model was then used to subtract out the mortality rate of the emulsion carrier. The median lethal dose of BDE-209 yolk-injected into chicken embryos was then calculated. This is a toxicological metric often used in risk assessment and policy decision making.

RESULTS

R.1: Trial 1: Testing the Effect of Egg Injection (Emulsion or DI Water) on Embryonic Mortality, December 2007

This was the preliminary trial of the yolk injection methodology conducted at VIMS. Embryos were sacrificed, by design, prior to hatching. A total of 13 eggs were used in this trial. The weight losses of the eggs during the incubation were slightly greater than optimal, yet not statistically different, see Appendix A: Figure A.1.

The mortality data are presented in Table R.1. The null hypothesis tested here was that yolk injection (DI water or emulsion) would have no effect on embryonic mortality. The Chi-Square value was equal to 10.19 with a corresponding $P<0.05$. There was a significant treatment effect on survival. It is hypothesized that the yolk-injections of DI water caused osmotic shock and embryonic death. The DIH2O treatment group was excluded from the analysis of the remaining data, as subsequent BDE-209 exposures did not involve DI water as a carrier, Using the remaining data, a Chi-Square value of 0.99 was calculated with a resulting p-value of 0.32. There was no significant difference in the mortality between the non-injected and the emulsion injected embryos in this small trial.

Table R.1: The Mortality Data for Trial 1

	Survive	Dead	Total	Percent Mortality
Non-inject	4	0	4	0
DIH2O-inject	0	4	4	100
Emulsion-inject	4	1	5	25
Total	8	5	13	38

On Day 19 of incubation, the embryos were sacrificed and necropsies were conducted. None of the embryos had begun to pip through the eggshell. The embryos were fully feathered. However, the yolk sacs were still external. The summary statistics for this trial are shown in Table R.2.

Table R.2: Summary Statistics for Trial 1

Treatment	Egg Wt (g)	Crown to Rump Length (in)	Body Wt w/yolk (g)	Yolk Sac Wt (g)	Liver Wt (g)	Brain Wt (g)
Vehicle-Inject Mean	58.67(4.14)	3.52(0.21)	44.60(8.12)	16.67(6.01)	0.59(0.14)	0.67(0.12)
Non-Inject Mean	59.07(3.46)	3.57(0.21)	49.57(2.8)	22.12(1.01)	0.55(0.06)	0.66(0.09)
Mean of all	58.84 (3.55)	3.55(0.20)	47.09(6.22)	19.40(4.99)	0.57(0.10)	0.66(.10)

Standard deviations reported in ().

The null hypothesis tested was that there were no differences between the various measurements in the two treatment groups. One-way ANOVA and follow up Tukey's Test were run on the above measurements. No significant differences were detected between the emulsion-injected and the non-injected groups.

R.2: Trial 2: Testing the effect of yolk-injection on embryonic mortality, (February 2008)

In this trial an automated egg turner was used. A sham injection treatment group was substituted for the DI water injection treatment to examine the impact of the physical trauma associated with the injection process. The mortality data for Trial 2 are summarized in Table R.3. The null hypothesis tested here was that there was no difference in mortality between eggs injected with the emulsion, sham injected, or non-injected (not-drilled) eggs. The Chi-Squared value calculated for this trial was 1.7 with a corresponding p value of 0.43. This suggests that there were no significant differences in mortality between the three different treatments. However, mortalities for all treatments were high, ranging from 25% to 66%.

The moisture loss data for this trial are presented in Appendix A Figure A.2 . The moisture weight loss was less than optimal, although again not statistically different.

Table R.3: Mortality Data for Trial 2

	Survive	Dead	Total	Percent Mortality
Non-inject	12	3	15	20
Sham-inject	9	6	15	40
Emulsion-inject	9	6	15	40
Total	30	15	45	33

R.3: Trial 3: Testing the effects of a single BDE-209 dose on embryonic mortality, June 2008

In this trial three treatments were examined: an 80 μg/egg BDE-209 dose, vehicle, and non-injected control. Mass mortality of the BDE-209 injected group occurred within two days of treatment. The mortality data for this trial are presented in Table R.6. The Chi-Squared calculated from this table equaled 139.8 ($P<0.001$). Again the data suggested an impact of yolk injection on embryonic mortality. By removing the non-injected control group from the analysis, we can determine if there is a statistical difference between the impact of the emulsion vehicle on embryonic mortality versus the impact of the BDE-209. Contingency analysis performed on these data yielded a Chi-Square of 103.8 ($P<0.001$). There was a significant impact on embryonic mortality when BDE-209 was injected into the yolk, compared to the vehicle emulsion itself. BDE-209 was toxic to embryonic chickens at a dose of 80 μg/egg.

This is the first trial that was run at the PWRC. PWRC performs regular egg incubation studies and has superior equipment to monitor and control incubator humidity. The moisture loss data for this trial are presented in Appendix A Figures A.3 through

A.5. Figure R.6 tracks the moisture weight loss of the BDE-209 injected eggs. Of the three treatment groups, the BDE-209 injected group deviated the most from the ideal moisture weight loss function, likely due to the fact that almost all (77 of 79) died following the injection. Only two embryos survived through to Day 19. The ideal moisture weight loss function was determined to be 16% of the Day 0 weight at hatch, day 21. The mean of all eggs within a treatment group on day 0 was multiplied by 0.84 to determine the ideal average weight at hatch, day 21. The two points were then plotted and a line was drawn to give the ideal moisture weight loss function. Therefore, an average of 79 egg weights was used to determine the function shown in Figure R.6. Only 2 eggs were actually tracked through to the completion of the study.

Table R.4: Mortality Data from Trial 3

	Survive	Dead	Total	Percent Mortality
Non-inject	38	1	39	3
Vehicle	66	14	80	17
Deca	2	77	79	98
Total	106	92	198	47

R.4: Trial 4: Testing the effect of multiple doses of BDE-209 on embryonic mortality, July 2008.

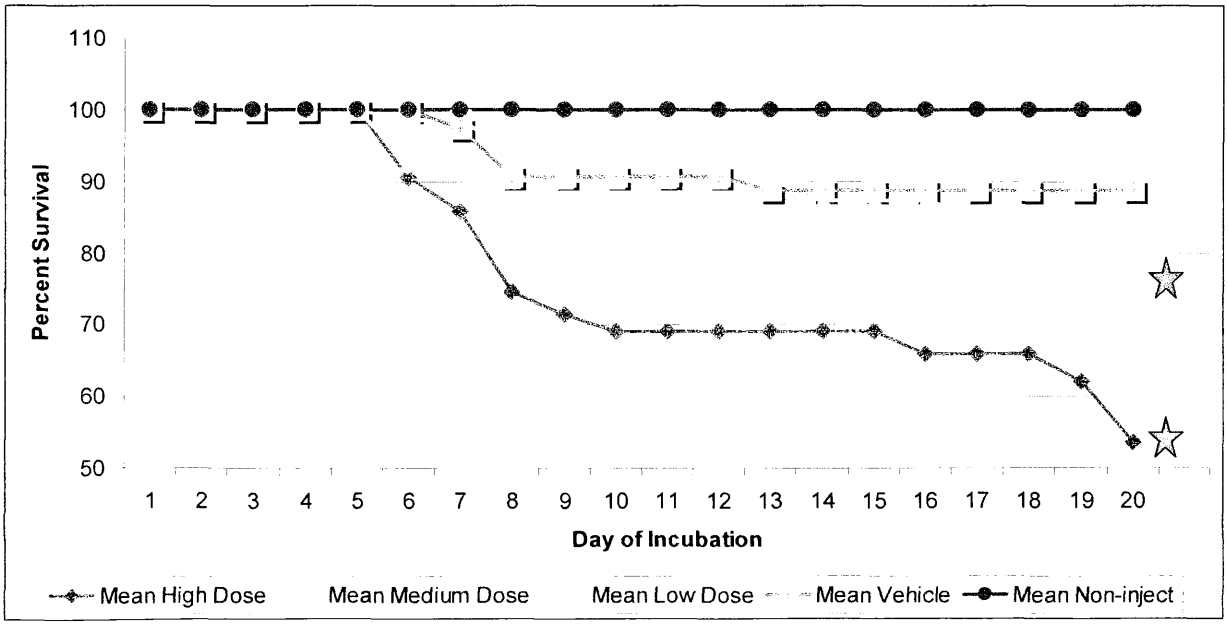
Three BDE-209 doses were injected into chicken yolks. The injection volume was 50 μL. The high dose was 40 μg/egg or 800 μg/mL, the middle dose 20 μg/egg or 400 μg/mL and the low dose 5 μg/egg or 100 μg/mL. The mortality data from this trial

are presented in Table R.5. The null hypothesis tested was that the various doses of BDE-209 injected into the yolks would have no affect on embryonic mortality. The Chi-Squared value for this table equaled 34.5 (df = 4) with P<0.001. There was a clear dose response relationship, as shown in Fig R.7.

Table R.5: Mortality Data from Trial 4

	Survive	Dead	Total	% Mortality
Non-Inject	30	0	30	0
Vehicle	39	4	43	9.3
Low Dose	42	8	50	16
Mid Dose	37	11	49	22
High Dose	24	23	47	49
Total	172	46	219	

Figure R.1: Tracking Mortality of 5, 20 or 40 µg/egg doses of BDE-209 in embryonic chickens versus vehicle control and non-injected treatments



☆ Significantly different from the Vehicle Control at α of 0.05

R.4.1Analysis of Tissue Weights from Trial 4

On Day 20 of Trial 4 the viability of the eggs in all of the treatment groups were evaluated using the Vetronic Buddy instrument.. All embryos that were alive on the morning of Day 20 were set to be removed from the eggs and sacrificed by decapitation. The Middle Dose (n = 41) and the Vehicle (n = 40) treatment groups were necropsied and various tissue weights were collected. Mortality occurred on Day 20 between the morning measurements and later sacrifices in both of these treatment groups. In the Middle Dose treatment group 4 embryos were dead at the time of removal from the egg. In the Vehicle treatment group, 5 of the embryos were dead at the time of removal from the eggs. Following death in the egg, the embryonic tissues begin to fill with fluid. The resulting edema skewed the weight measurements taken from embryos that died before removal from the egg shell. Therefore the data from these individuals were excluded from the following analysis of the tissue weights.

Table R.6 presents the tissue weight data from Trial 4. Two-sample T-Tests were run to determine if BDE-209 had any impact on tissue weights. The null hypotheses were that there are no differences between the mean tissue weights from the two treatment groups. At α=5%, none of the treatments were significantly different.

Often data for individual tissues are expressed as ratios relative to the total body weight instead of as raw values. The calculation to convert the raw value to the tissue index is shown below.

$$TissueIndex = \frac{TissueWeight}{TotalBodyWeight} * 100$$

The transformed tissue data are presented in Table R.7. Two-sample T-Tests were run to determine if BDE-209 had any impact on tissue weights. The null

hypotheses were that there are no differences between the mean tissue indices from the two treatment groups. Again, at α= 5% none of the treatments were significant.

Table R.6: Mean (Standard Deviation) Tissue Weights from Trial 4

TREATMENT	Body wt w/ yolk (g)	Yolk (g)	Liver (mg)	Brain (mg)	Muscle (mg)	Sample size
VEHICLE	41.0 (3.68)	10.5(2.06)	570 (107)	867 (68.6)	178 (28.4)	35
DECA MID DOSE	39.8(3.36)	10.1(1.71)	590 (81.2)	877 (62.0)	187 (26.7)	37

Table R.7: Mean (Standard Deviation) Tissue Indices from Trial 4

TREATMENT	Liver Index	Brain Index	Heart Index	Sample size
VEHICLE	1.8 7(0.35)	2.84 (0.32)	0.58 (0.10)	35
DECA MID DOSE	1.99 (0.24)	2.96 (0.30)	0.63 (0.08)	37

R.5: Dose Verification

The high dose was extracted efficiently with recoveries of 85-98%. The middle dose had lower extraction efficiency with recoveries ranging from 51-79%. The low dose extractions yielded much lower concentration estimates than expected, i.e. 19, 39 and 52% recovery. The expected concentration of the diluted low dose extractions was lower (983 ng/mL) than the medium and high doses (1600 ng/mL). As expected, BDE209 was not detected in the vehicle extractions. These extracts were much more concentrated than the others, which required substantial dilution to allow BDE-209 quantitation.

Table R.8: Dose Verification

Sample	BDE209 (ng/ml)	Expected BDE209 (ng/ml)	% Rec. BDE209
HIGHDOSEREP1	1430	1600	89
HIGHDOSEREP2	1570	1600	98
HIGHDOSEREP3	1370	1600	86
MEAN (SD)	**1450(100)**		**91 (6.4)**
MEDDOSEREP1	830	1600	52
MEDDOSEREP2	1120	1600	70
MEDDOSEREP3	1270	1600	79
MEAN (SD)	**1070(223)**		**67 (14)**
LOWDOSEREP1A	186	983	18
LOWDOSEREP2A	507	983	52
LOWDOSEREP3A	384	983	39
MEAN (SD)	**359(160)**		**36 (16)**
VEHREP1	ND	0	NA
VEHREP2	ND	0	NA
VEHREP3	ND	0	NA

The BDE-209 standard used in the making of the dosing formulations in this study was high purity, i.e. >98%. Does not look that high in your below figure However, the presence of small amounts of nona-BDEs in the BDE-209, and therefore the dosing solutions, can not be ignored; especially as this study aims to quantify any biological debromination that may have occurred. Two of the nona-BDEs (-207 and 206) were identified and quantified to account for the entire mass of the powder dissolved in each dosing formulation. When the nona-BDEs are taken into account for the medium dose dilutions the recoveries improve to 70.5%, 85.9%, and 96.7%, respectively. BDE-206 was more abundant than BDE-207. In the medium dose extractions BDE-206 represented on average 15% of the total with BDE-207 contributing 5% of the total composition.

Figure R.2: Medium Dose Verification

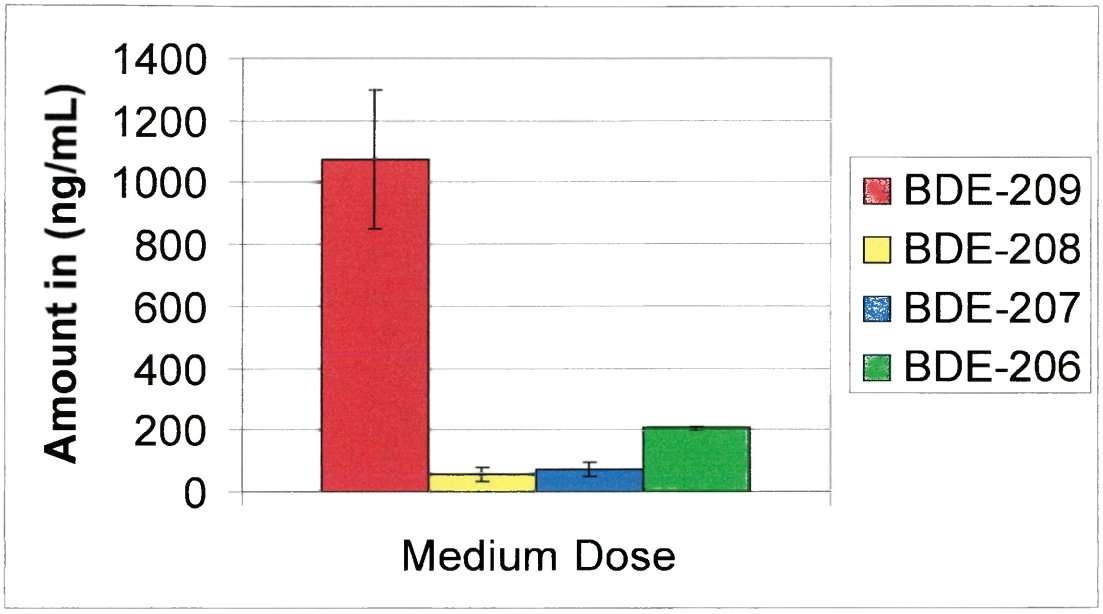
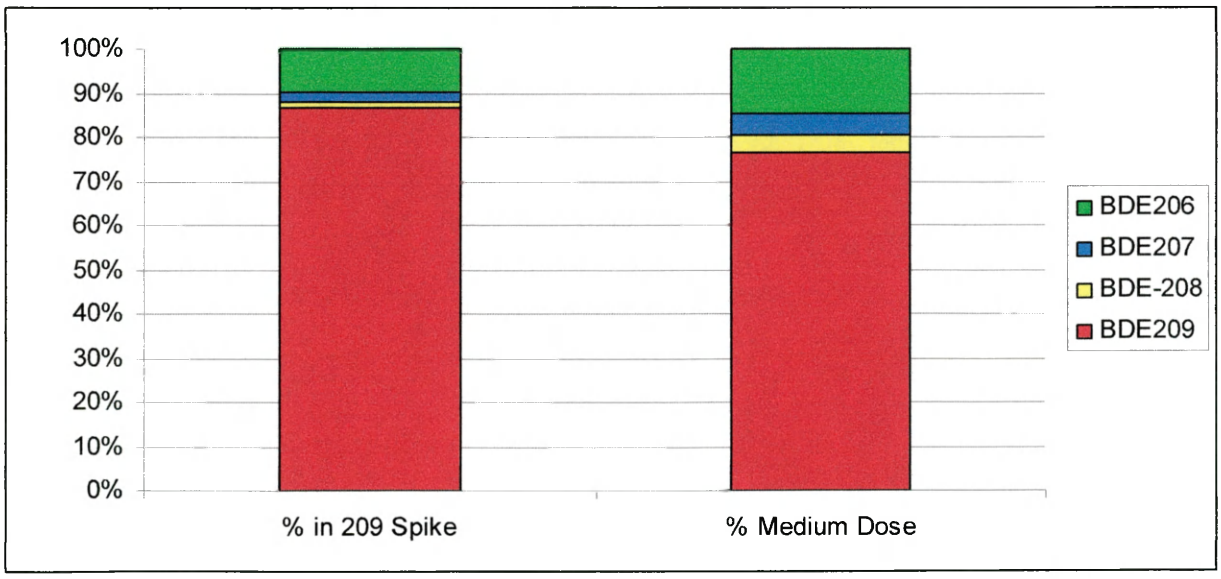


Figure R.3: PBDE Congener Profiles for the Middle Dose and Spiking Solution



R.6: Calculating the LD50

The Median Lethal Dose (LD50) was determined here by following the methods presented in the Newman text from 1995. Figures R.7 through R.9 present the SAS readouts for the Probit, Logit, and Gompertz Analyses. The Gompertz model had the lowest Pearson Chi-Square value. Therefore, the data are a better fit to the Weibull distribution. Figures R.4 through R.6 revealed that the three models all generated equivalent values for the LD50.

The three models (Probit, Logit, and Gompertz) include a term to account for the mortality caused by the injection of the vehicle alone. It should be noted here that data from trials three and four were used to conduct this analysis. Two different injection volumes were used. These data were only pooled together after careful consideration. The importance of the LD50 metric in toxicology and risk assessment is that it is widely used and understood. By pooling the data from two trials, this metric contains more information on the toxicity of this compound to embryonic birds. However, the trade off is that the information on the toxicity of the emulsion vehicle is less accurate. The reason for this is that in trial three 100 µl of vehicle were injected and in trial four only 50 µl of the vehicle were injected. Therefore the vehicle mortality rate of 16.8% is likely overestimated for the 50 µl injections and is underestimated for the 100 µl.

All three of the models calculated an LD50 of 44 µg/egg for a 16-day *in ovo* exposure. The estimated mortality rate from exposure to the emulsion vehicle ranged from 16-18%.

Figure R.4: SAS Readout for Probit Analysis

Probit Analysis (assuming a normal distribution)

Goodness-of-Fit Tests

Statistic	Value	DF	Pr > ChiSq
Pearson Chi-Square	2.3325	2	0.3115
L.R. Chi-Square	2.1717	2	0.3376

Pearson Chi-Squared

Analysis of Parameter Estimates

Parameter	DF	Estimate	Standard Error	95% Confidence Limits		Chi-Square	Pr > ChiSq
Intercept	1	-11.4285	2.6205	-16.5645	-6.2924	19.02	<.0001
Log10(CONC)	1	6.9646	1.5074	4.0101	9.9191	21.35	<.0001
_C_	1	0.1827	0.0286	0.1268	0.2387		

Estimate of natural mortality rate

Probit Procedure

Probit Analysis on CONC

Probability	CONC	95% Fiducial Limits	
0.45	41.96547	32.79999	48.00176
0.50	43.74565	34.99876	49.78320
0.55	45.60136	37.28541	51.71317

LD50 = 43.75 ug/egg

Figure R.5: SAS Readout for Logit Analysis

Logit Model (Assumes a Logistic distribution)

Goodness-of-Fit Tests

Statistic	Value	DF	Pr > ChiSq
Pearson Chi-Square	2.1100	2	0.3482
L.R. Chi-Square	1.9738	2	0.3727

Pearson Chi-Squared

2.11 < 2.33

The logit model fits better than the probit model

Analysis of Parameter Estimates

Parameter	DF	Estimate	Standard Error	95% Confidence Limits		Chi-Square	Pr > ChiSq
Intercept	1	-20.4372	4.9116	-30.0638	-10.8106	17.31	<.0001
Log10(CONC)	1	12.4847	2.9078	6.7855	18.1839	18.43	<.0001
C	1	0.1815	0.0284	0.1259	0.2372		

Estimate of natural mortality rate

Probit Procedure

Probit Analysis on CONC

Probability	CONC	95% Fiducial Limits	
0.45	41.77389	34.15946	47.26338
0.50	43.34891	36.15814	49.02738
0.55	44.98332	38.17955	50.98271

LD50 = 43.34 ug/egg

Figure R.6: SAS Readout for Gompertz Analysis

Gompertz Model (Assumes an exponential/Weibull distribution)

Goodness-of-Fit Tests

Statistic	Value	DF	Pr > ChiSq
Pearson Chi-Square	0.5129	2	0.7738
L.R. Chi-Square	0.5007	2	0.7785

Pearson Chi-Squared

0.5129 < 2.11

The Gompertz model fits better than the logit model

Analysis of Parameter Estimates

Parameter	DF	Estimate	Standard Error	95% Confidence Limits		Chi-Square	Pr > ChiSq
Intercept	1	-10.5079	2.0225	-14.4719	-6.5439	26.99	<.0001
Ln(CONC)	1	2.6783	0.4848	1.7281	3.6285	30.52	<.0001
_C_	1	0.1682	0.0295	0.1103	0.2261		

Estimate of natural mortality rate

Probit Procedure

Probit Analysis on CONC

Probability	CONC	95% Fiducial Limits	
0.45	41.73140	31.63374	48.68537
0.50	44.10107	34.29392	50.95795
0.55	46.49370	37.01070	53.28432

LD50 = 44.10 ug/egg

Total egg weight changed throughout the course of incubation (~16%) due to moisture weight loss. The mean initial egg weight was 59.7 g. This yields an LD50 concentration of 740 ng/g egg. If one corrects this value for ideal moisture weight loss of 16% then the value rises to 880 ng/g egg.

In examining the potential toxic impact of these levels of BDE-209 on real world bird populations, different lethal dose metrics may be examined. Again the accuracy of these numbers decreases as we move away from the middle (LD50) of the CDF. The LD10 calculated by the Gompertz model in this study is 21.8 μg/egg. This equates with 365 ng/g egg.

R.7: BDE-209 Egg Distribution and Biotransformation

R.7.1: Method Development

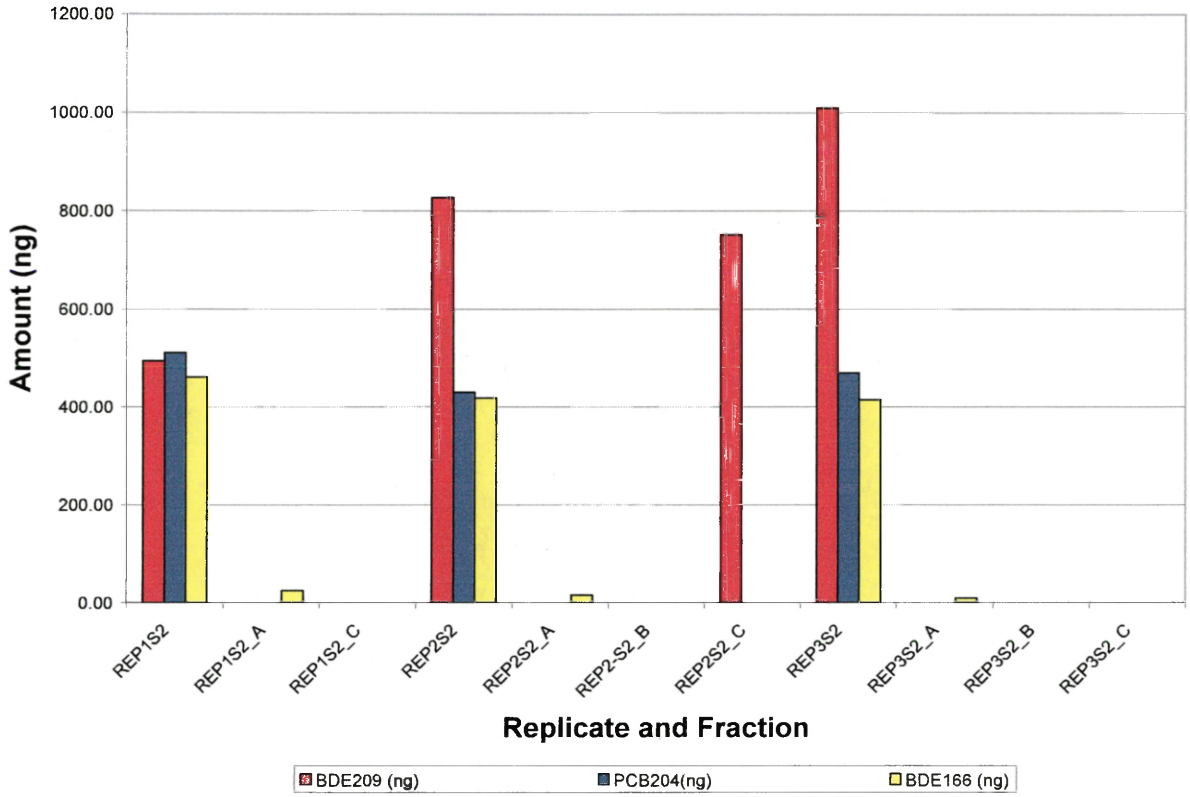
The solid-phase extraction (SPE) step has been used in the VIMS lab as a viable step for eliminating co-extracted biogenics from a variety of matrices. However, preliminary analysis by GC/MS of some egg extracts suggested that excessive biogenic interferences remained in the purified extract if the solvent regime typically used was employed. Weaker solvent regimes might elute off less material, so alternative column elution options were examined. Initially only 100% hexane was used. BDE-209 was eluted, but required a high volume (15 ml) of solvent. During these trials, it became apparent that the interaction of bromine with the silica gel column caused the PBDEs to elute off the column in an unexpected manner. BDE-209, the fully brominated congener, was expected to be the last of the PBDE congeners to elute from the silica gel column. However, BDE-209 was actually the first PBDE to elute.

R.7.2: 10% DCM SPE test

A solvent regime of 10% DCM and 90% hexane was next tested. Three replicates were run. The column was prepped with 5 ml of 100% hexane. The SPE columns were spiked with 1000 ng BDE209, 480 ng PCB204, and 480 ng BDE166. The first fraction was eluted with 3 ml of hexane and discarded. The second (S2) fraction consisted of 8 ml of 10% DCM 90% hexane. This was collected, reduced to < 1ml and spiked with 300 ng DCDE as internal standard before GC/MS analysis. The third (S2_A) fraction consisted of an additional 3 ml of 10% DCM. The fourth and fifth fractions (S2_B and S2_C, respectively), were also 3 ml of 10% DCM. These were also reduced to <1ml and spiked with 300 ng of DCDE as an internal standard. In all three replicates BDE-166

appeared in the S2_A fraction. The appearance of BDE-209 in the S2_C fraction of replicate 2 was unexpected and may be due to contamination.

Figure R.7: Data from the SPE 10% DCM Test



R.7.3: PBDE Spike Recovery Study

The results of the egg spiking experiments indicated the recoveries of all the compounds except BDE-47 and PCB-204 exceeded 80% (Table R.6). Some BDE-47 may still have been retained on the SPE after elution. The overall focus of the project was on BDE209 and the most likely degradates (nona- and octa- PBDE congeners), so some loss of low brominated congeners, e.g. tetrabrominated BDE-47, was deemed acceptable, especially in the face of greater elution of chromatographically co-eluting biogenic interferences.

Table R.9: Spike Recovery Test of PBDEs from sodium sulfate (B-FL), Food Lion whole egg (FLWE) and yolk (FLY) samples.

	BDE209 (ng/ml)	% Rec BDE209	BDE166 (ng/ml)	% Rec BDE166	BDE154 (ng/ml)	% Rec BDE154	BDE183 (ng/ml)	% Rec BDE183	BDE99 (ng/ml)	% Rec BDE209	BDE47 (ng/ml)	% Rec BDE47
B-FL-5	1240	110.2	453	98.2	989	87.9	1170	104.0	1100	97.8	720	64.0
B-FL-6	1090	96.9	416	90.2	1230	109.3	1070	95.1	1180	104.9	1160	103.1
B-FL-7	1200	106.7	393	85.2	989	87.9	1080	96.0	1140	101.3	760	67.6
Blank mean		104.6		91.2		95.0		98.4		101.3		78.2
FLWE-6	106	94.2	567	122.9	1000	88.9	1280	113.8	1160	103.1	496	44.1
FLWE-7	1100	97.8	584	126.6	936	83.2	1290	114.7	1140	101.3	389	34.6
FLWE-8	1130	100.4	513	111.2	963	85.6	1140.	101.3	1200	106.7	507	45.1
Egg mean		97.5		120.2		85.9		109.9		103.7		41.2
FLY-6	1150	102.2	510	110.6	1060	94.2	1150	102.2	1250	111.1	691	61.4
FLY-7	1290	114.7	525	113.8	906	80.5	1140	101.3	1200	106.7	537	47.7
FLY-8	1330	118.2	638	138.3	976	86.8	1210	107.6	1250	111.1	438	38.9
Yolk mean		111.7		120.9		87.2		103.7		109.6		49.4
Mean of all		104.6		120.6		89.4		104.0		104.9		56.3

R.7.4: QA/QC

Sodium Sulfate (Blanks) samples were run with each pool of 10 samples. The data from these samples are presented in Table R.10. BDE-209 was not detected in any of these blank samples.

$$LimitofQuantitation = \frac{164ng}{(1g)*(0.5625)} = 290ng/g$$

Blank 3 shows a very low recovery of BDE-166. The sample was re-run and the results were the same. The peak for BDE-166 was definitely present yet the concentration was too low to quantify.

Table R.10: Sodium Sulfate Spiked Blank Samples

Sample ID	BDE209 (ng/ml)	BDE166 (ng/ml)	% REC
BLANK-2	ND	351	62.4
BLANK-3	ND	<290	NA
BLANK-4	ND	505	101.0
BLANK-5	ND	330	66.0
BLANK-6	ND	409	81.8
BLANK-7	ND	485	97.0
BLANK8	ND	632	126.4
BLANK-2 RERUN	ND	417	74.1
BLANK-3 RERUN	ND	<290	NA
BLANK-5 RERUN	ND	328	58.3
BLANK-8 RERUN	ND	471	83.7

R.7.5: Results from Distribution Study

Tables R.11 through R.20 summarize the data from the 80 injected egg samples collected in the BDE-209 distribution study. These tables are organized by tissue type and treatment group. The samples are named by tissue type (i.e. B = brain, C = carcass, Y = Yolk, L = Liver, H = Heart) then treatment (V = Vehicle D = Dosed) and the number of the sample pool. Each pool number equates with the same four birds, e.g. sample BD4 contains the

brains of the birds whose carcasses can be found in sample CD4. This allows for both analysis of tissue mean values and individual pool values.

The quality of the data is evaluated by the percent recovery of the surrogate standard BDE-166. Any samples where the percent recovery of the surrogate standard was >120% or <70% or were rerun on the GC/MS. The data from the reruns are reported in the appropriate table.

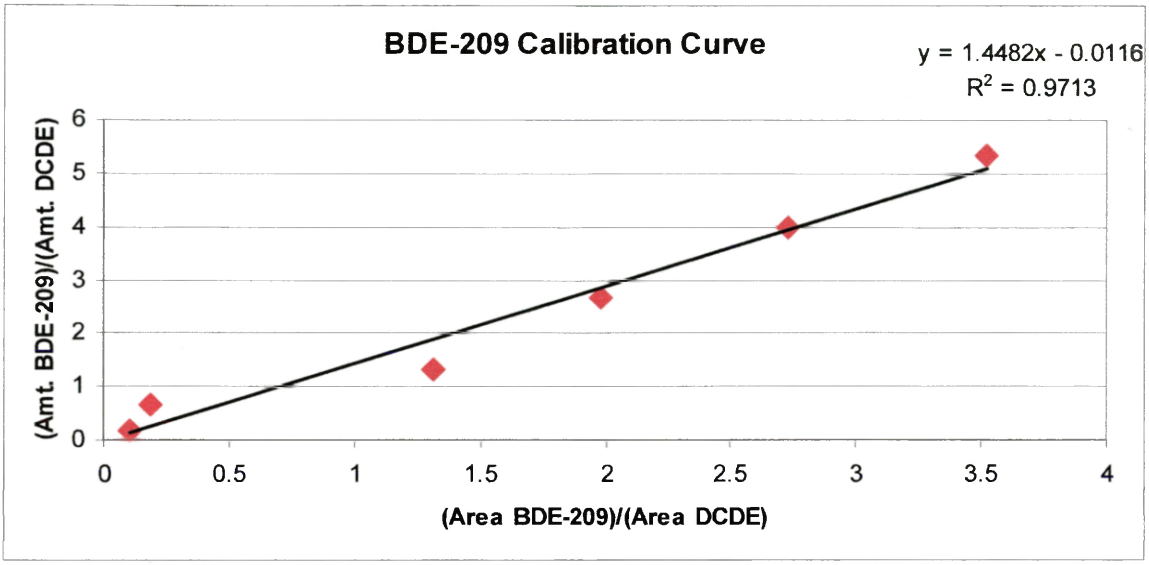
Note that the third column in each table entitled "Corrected [BDE-209] (ng/g lw)" contains the following formula:

$$CorrectedConcentration[BDE-209] = \frac{(AmountBDE-209) \div (0.5625)}{lipidwt} \div (BDE-166\%\,\mathrm{Recovery})$$

The constant 0.5625 is the percentage of the ASE sample either not used in lipid analysis (10% of the sample is consumed in the gravimetric determination of the sample lipid content) or un-injected in the size exclusion process. In the SEC step the total sample volume is diluted to 8 ml and the sample is split in two for loading onto the HPLC. The sample volume charged to the SEC column actually occurs as two injections; one from each 4 ml sample aliquot. Each injection is only 2.5 ml. So a total of 5 ml of the 8 ml total sample reaches the column. This is a ratio of 5/8 of the original sample AFTER 10% has been removed for lipid analysis, hence the 0.5625 factor.

The calibration curve used to quantify BDE-209 (Figure R.8) ranged from 50 ng/ml BDE-209 to 2000 ng/ml BDE-209. This curve was linear with an R-Squared value of 0.9713.

Figure R.8: BDE-209 Calibration Curve



R.7.5.1: Brain Samples

BDE-209 was not detected in any of the brain samples from the vehicle treatment group. (Table R.12) BDE-209 was detected at high levels in only one of the original brain samples from the dosed treatment group. (Table R.11) This sample (BD8) was rerun (BD8-R) and still exhibited high BDE-209 concentrations. BDE-166 recovery was 91%. It is unlikely that such high levels would be detected in only one of the original dosed brain samples. It is much more likely that this large BDE-209 signal represents cross-contamination in the laboratory.

After initial analysis the dosed brain samples were reduced in volume and pooled together from a total of 7 individual samples (BD8 was not pooled) into two samples. These composite samples were then analyzed by GC/MS. The results are presented in Table R.11A.

Table R.11: BDE-209 in Brain Samples from Middle Dose and Vehicle Yolk-injected Eggs

Sample ID	Corrected [BDE209] (ng/g ww)	Corrected [BDE209] (ng/g lw)	BDE166 (ng)	% REC	% lipid
BD2	ND	ND	453	81	28
BD3	ND	ND	409	73	30
BD4	ND	ND	393	79	27
BD5	ND	ND	338	68	30
BD6	ND	ND	484	97	26
BD7	ND	ND	381	76	28
BD8	540	9400	551	110	35
BD8-R*	380	6600	514	91	35
			MEAN	83	30
			STDEV	15	3.4
BV2	ND	NA	482	86	34
BV3	ND	NA	373	66	39
BV3-R*	ND	NA	271	48	39
BV4	ND	NA	541	108	13
BV5	ND	NA	401	80	96
BV6	ND	NA	592	118	29
BV7	ND	NA	583	117	28
BV8	ND	NA	518	104	4.8
			MEAN	97	35
			STDEV	20	25

* The –R indicates this is a rerun of the sample. "B" indicates Brain Sample, "D" indicates from the middle dosed group, "V" indicates from the vehicle control group. The number in the sample ID refers to the specific samples pool.

Table R.11A: BDE-209 in Composite Brain Samples from BDE-209 –injected Eggs

	BDE209 ng/g dw	BDE166	%Recovery BDE-166
NEWBRAIN1	56	1920	114
NEWBRAIN2	ND	2150	127

*NEWBRAIN1 contains samples BD1, BD2, BD3, and BD4
*NEWBRAIN2 contains samples BD5, BD6, and BD7.
BD8 was not pooled due to the high levels of BDE-209 present.

R.7.5.2: Carcass Samples

Each carcass sample extracted had a dry weight of 1.5 grams. The average lipid weight of the carcass samples was 0.405 g. The lowest point on the GC/MS calibration curve was 50 ng/ml. The calculations to determine the limit of quantitation are shown below.

$$Limit of Quantitation = \frac{50ng}{(0.405g)*(0.5625)} = 219ng/glw$$

BDE-209 was not detected in any of the vehicle exposed carcass samples, see Table R.13. BDE-209 was detected in all dosed carcass samples, see Table R.14. The mean concentration of BDE-209 in the dosed samples was 574 ng/g lw.

Table R.12: BDE-209 in Carcasses from Middle Dose and Vehicle-injected eggs

	Corrected [BDE209] (ng/g ww)	Corrected [BDE209] (ng/g lw)	BDE166	% REC	% lipid
CD2	27.0	509.8	429	76.3	27.1
CD3	26.4	529.5	459	81.6	25.4
CD4	18.9	345.8	470	83.6	27.9
CD5	18.7	355.2	416	74.0	26.9
CD6	26.8	534.2	585	104.0	25.6
CD7	25.8	510.6	416	74.0	25.8
CD8	65.7	1235.4	533	94.8	27.1
MEAN	**29.9**	**574.4**	**MEAN**	**84.0**	**26.6**
STDEV	**16.2**	**302.5**	**STDEV**	**10.6**	**0.9**
CV2	ND	ND	511	90.8	27.5
CV3	ND	ND	418	74.3	27.3
CV4	ND	ND	476	95.2	26.5
CV5	ND	ND	385	77.0	27.5
CV6	ND	ND	589	117.8	26.9
CV7	ND	ND	443	88.6	23.6
CV8	ND	ND	563	112.6	32.6
			MEAN	93.8	27.4
			STDEV	16.5	2.7

"C" indicates Carcass Sample, "D" indicates from the middle dosed group, "V" indicates from the vehicle control group. The number in the sample ID refers to the specific samples pool.

R.7.5.3: Liver Samples

The amount of sample available from the pooled livers averaged 0.78 g. The average lipid weight of the liver samples was 0.39 g. The calculations to determine the limit of quantitation for these samples are shown below.

$$Limit of Quantitation = \frac{50ng}{(0.39g) * (0.5625)} = 227ng / glw$$

BDE-209 was detected in only one of the vehicle-exposed liver samples, see Table R.16. This sample, LV7, was identified as an outlier and was rerun. LV2 was dropped during processing and lost. BDE-209 was detected in all dosed liver samples, see Table R.17.

Three of these samples have recoveries below 70% for BDE-166 and were rerun

The mean concentration of BDE-209 in the dosed liver samples was 3640 ng/g lipid weight.

Table R.13: BDE-209 in Liver from Middle Dose and Vehicle-injected Eggs

Sample ID	Corrected [BDE209] (ng/g ww)	Corrected [BDE209] (ng/g lw)	BDE166	% REC	% lipid
LD2	749.3	5307.8	393	69.9	38.8
LD2-R*	225.8	1599.7	283	50.3	38.8
LD3	743.8	4690.6	372	66.1	43.6
LD3-R*	209.1	1318.8	648	115.2	43.6
LD4	749.5	4810.5	449	89.8	42.8
LD5	971.7	2222.2	326	65.2	120.1
LD5-R*	313.6	717.2	352	70.4	120.1
LD6	893.3	5745.0	533	106.6	42.7
LD7	679.4	5294.0	493	98.6	35.3
LD8	691.6	4658.9	497	99.4	40.8
MEAN	**622.7**	**3636.5**	**MEAN**	**83.2**	**56.6**
STDEV	**273.6**	**1931.1**	**STDEV**	**21.5**	**33.6**
LV3	ND	ND	664	118.0	29.0
LV4	ND	ND	409	81.8	35.6
LV5	ND	ND	320	64.0	47.4
LV5-R*	ND	ND	269	53.8	47.4
LV6	ND	ND	566	113.2	40.8
LV7	128.000633	927.9895732	453	90.6	37.9
LV8	ND	ND	505	101.0	36.4
			MEAN	88.9	39.2
			STDEV	24.1	6.6

* The –R indicates this is a rerun of the sample. "L" indicates Liver Sample, "D" indicates from the middle dosed group, "V" indicates from the vehicle control group. The number in the sample ID refers to the specific samples pool.

The nona BDEs were detected in all of the dosed liver samples. The data are presented in Table R.14.

Table R.14: Nona-BDEs in Dosed Liver samples

SAMPLE ID	Corrected [BDE206] (ng/g lw)	Corrected [BDE207] (ng/g lw)	Corrected [BDE208] (ng/g lw)
LD1	104.1	129.9	22.0
LD2	175.2	245.2	108.8
LD3	225.0	219.4	64.9
LD4	376.2	443.2	152.0
LD5	135.2	114.7	37.8
LD6	529.1	574.7	125.6
LD7	613.4	674.5	269.3
LD8	198.1	303.1	86.6
Mean	**294.6**	**338.1**	**108.4**
STDEV	**190.3**	**206.1**	**78.3**

"L" indicates Liver Sample, "D" indicates from the middle dosed group, "V" indicates from the vehicle control group. The number in the sample ID refers to the specific samples pool.

R.7.5.4: Heart Muscle Samples

The amount of sample available from the pooled heart muscles averaged only 0.1 g. The average lipid weight of the heart muscle samples was 0.019g. The calculations to determine the limit of quantitation for these samples are shown below.

$$LimitofQuantitation = \frac{50ng}{(0.019g)*(0.5625)} = 4700ng/glw$$

BDE-209 was not detected in any of the vehicle exposed heart samples, see Table R.18. BDE-209 was also not detected in any of the original dosed heart samples, see Table R.19. The final pre-GC injection volume of these samples was 1 ml. The dosed heart samples were blown down and pooled together from 8 original samples into 2 new samples and rerun. The calculations for the new limit of quantitation are shown below.

$$LimitofQuantitation = \frac{50ng}{(0.019g*4)*(0.5625)} = 1700ng/glw$$

Table R.15: BDE-209 in Heart from Middle Dose and Vehicle-injected Eggs

Sample ID	Corrected [BDE209] (ng/g ww)	Corrected [BDE209] (ng/g lw)	BDE166	% REC	% lipid
MD2	ND	ND	429	76.3	20.0
MD3	ND	ND	393	69.9	14.0
MD3-R*	ND	ND	507	90.1	14.0
MD4	ND	ND	468	93.6	22.0
MD5	ND	ND	277	55.4	36.0
MD5-R*	ND	ND	398	70.8	36.0
MD6	ND	ND	574	114.8	18.0
MD7	ND	ND	453	90.6	18.0
MD8	ND	ND	715	143.0	18.0
MD8-R*	ND	ND	663	117.9	18.0
			MEAN	92.2	21.4
			STDEV	26.5	7.6
MV2	ND	ND	430	76.4	4.0
MV3	ND	ND	812	144.4	16.0
MV4	ND	ND	491	98.2	13.0
MV5	ND	ND	439	87.8	31.0
MV6	ND	ND	522	104.4	10.0
MV7	ND	ND	399	79.8	11.0
MV8	ND	ND	505	101.0	19.0
			MEAN	98.9	14.9
			STDEV	22.7	8.6

* The –R indicates this is a rerun of the sample. "M" indicates Heart Muscle Sample, "D" indicates from the middle dosed group, "V" indicates from the vehicle control group. The number in the sample ID refers to the specific samples pool.

Table R.15A: BDE-209 in Composite Heart Samples from BDE-209 injected Eggs

	BDE209 ng/g dw	BDE166	%Recovery BDE-166
NEWHEART1	245	1950	116
NEWHEART2	184	1980	117.

*NEWHEART1 contains samples MD1, MD2, MD3, and MD4
*NEWHEART2 contains samples MD5, MD6, MD7,and MD8.

R.7.5.5: Yolk Samples

Each yolk sample had a dry weight of 1.0 g. The average lipid weight of the yolk samples was 0.41g. The calculations to determine the limit of quantitation for these samples are shown below.

$$Lower\ \lim itofQuantitation = \frac{200ng}{(0.41g)*(0.5625)} = 867ng/glw$$

The highest point on the extended GC/MS calibration curve was 2000 ng/ml. The calculations to determine the upper limit of quantitation for the curve are shown below.

$$UpperLimitofQuantitation = \frac{2000ng}{(0.41g)*(0.5625)} = 8670ng/glw$$

BDE-209 was not detected in any of the vehicle-injected yolk samples, see Table R.19. The dosed yolk samples were all run in triplicate, as there was plenty of material available for analysis. BDE-209 was detected in all dosed yolk samples. The mean concentration of BDE-209 in the dosed samples was 6000 ng/g lw. Many of the detected values were above the upper limit of quantitation identified above.

The yolk samples were diluted 5-fold to bring the BDE-209 concentrations within the range of the calibration curve. These concentrations were found to be reasonable and well within the expected range given the total dose administered to the embryos. Therefore, further work with 13C-labeled BDE-209 was not required.

Table R.16: BDE-209 in Yolk from Vehicle-injected Eggs

Sample ID	Corrected [BDE209] (ng/g ww)	Corrected [BDE209] (ng/g lw)	BDE166 (ng/ml)	% REC	% lipid
YV2	ND	ND	437	77.7	32.2
YV3	ND	ND	371	66.0	38.7
YV4	ND	ND	496	99.2	45.8
YV5	ND	ND	516	103.2	44.5
YV6	ND	ND	429	85.8	42.8
YV7	ND	ND	536	107.2	43.6
YV8	ND	ND	507	101.4	48.9
			MEAN	91.5	42.4
			STDEV	15.4	5.4

"Y" indicates Yolk Sample, "D" indicates from the middle dosed group, "V" indicates from the vehicle control group. The number in the sample ID refers to the specific samples pool.

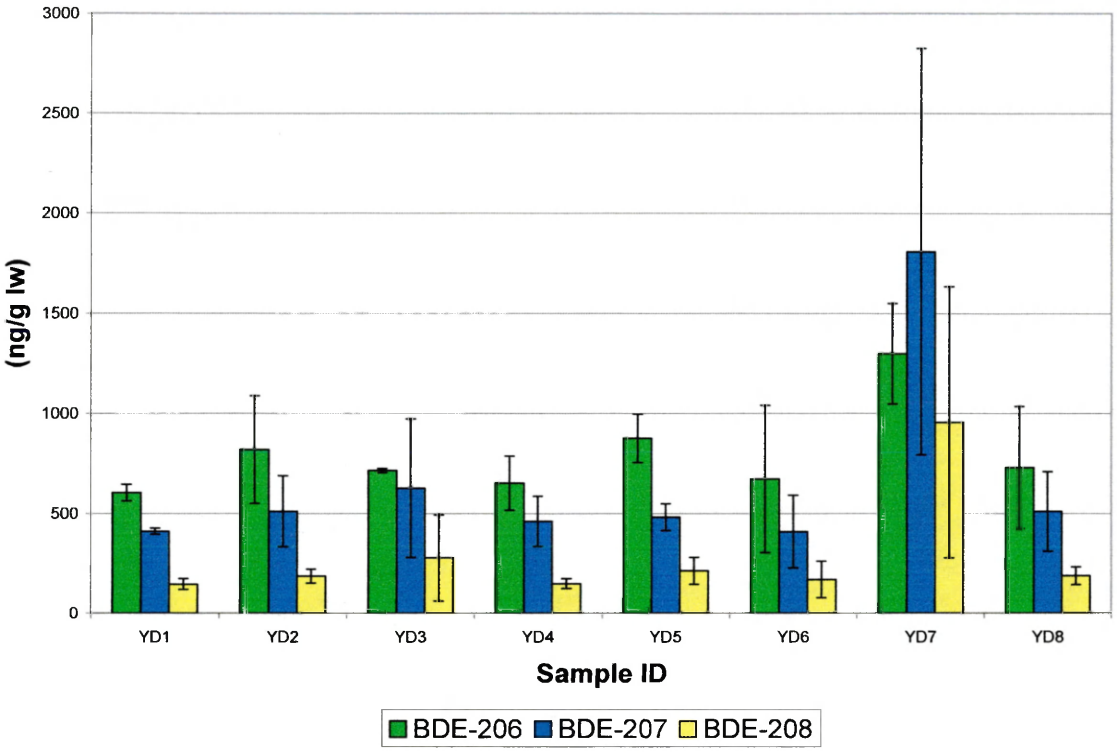
Table R.17 : BDE-209 in Diluted Yolk Samples from BDE-209-injected Eggs

Sample ID	BDE-209 (ng/g dw)	BDE-209 (ng/g lw)	% Recovery BDE-166
YD1*A5-1	2213	5533	49.07
YD2*A5-1	3520	8800	58.40
YD3*A5-1	3120	7800	61.78
YD4*A5-1	3947	9867	68.44
YD5*A5-1	3716	9289	45.07
YD6*A5-1	4098	10244	42.40
YD7*A5-1	4240	10600	69.51
YD8*A5-1	3858	9644	65.42
Mean	3589	8972	58
SD	656	1640	10.7

***A5-1 Refers to analytical replicate A diluted by a a factor of 5**. "Y" indicates Yolk Sample, "D" indicates from the middle dosed group, "V" indicates from the vehicle control group. The number in the sample ID refers to the specific samples pool.

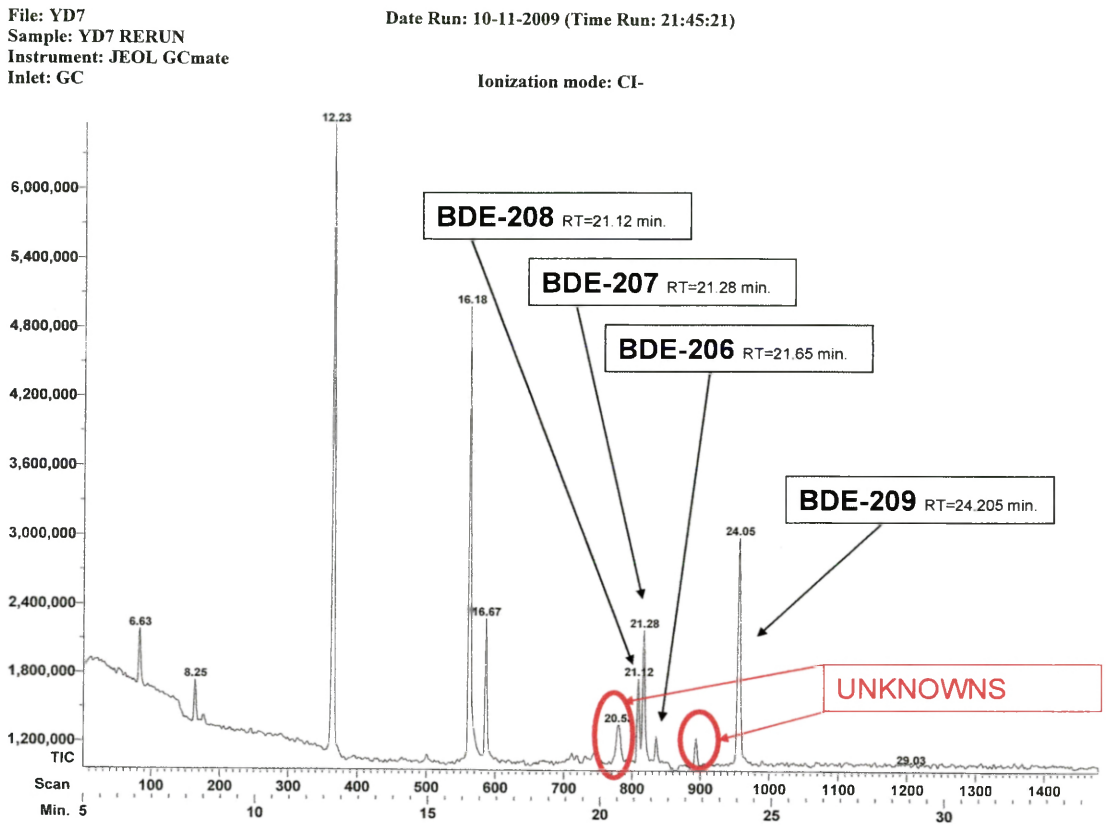
Nona BDEs were detected in all of the dosed yolk samples. Figure R.9 presents the mean values for the three nona-BDEs in the dosed yolk samples.

Figure R.9: Mean Nona-BDEs in Dosed Yolk Samples



Two unknown heavily brominated compounds were also detected in YD7 and YD3. The chromatograms and spectra for these samples are shown below in Figures R.10 through R.22.

Figure R.10: Chromatogram of Sample YD7



The spectra were compared to the spectra from La Guardia's ECNI BDE Spectra Library[5]. The two compounds with retention time 20.52 min. and 22.82 were not identified as PBDE congeners. They may be potential breakdown products of BDE-209 or contaminants in the analytical process. From the MS spectra, these two compounds are clearly brominated, due to the presence of the 79 and 81 m/z and with ion clusters at about 80 m/z intervals indicative of bromine losses.

The spectrum for Unknown Compound A is shown in Figure R.20. The 722 ion is indicative of a compound containing seven bromines (12 Carbons, 1 Oxygen, and 7

[5] http://www.vims.edu/people/laguardia_mj/pubs/LaGuardia_Spectra%20Library%20-%20ECNI%20PBDEs.pdf).

bromines). This is most likely a polybrominated dibenzofuran (PBDF) with eight bromines. The molecular ion was 800 with M-Br at 720 m/z. Figure R.21 shows the basic structure.

Figure R.11: Mass Spectrum of unknown Compound A with Retention Time of 20.52 min.

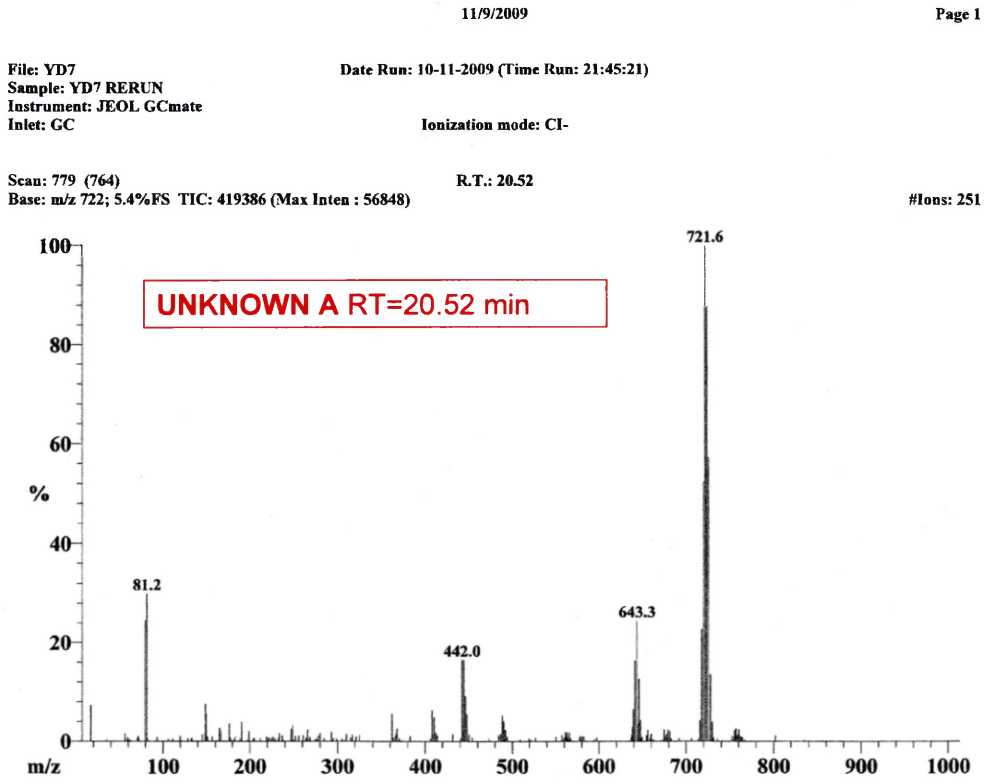
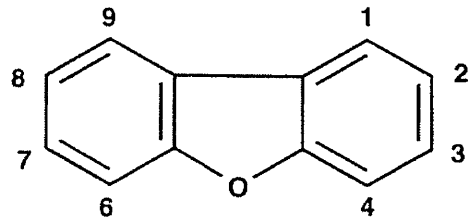


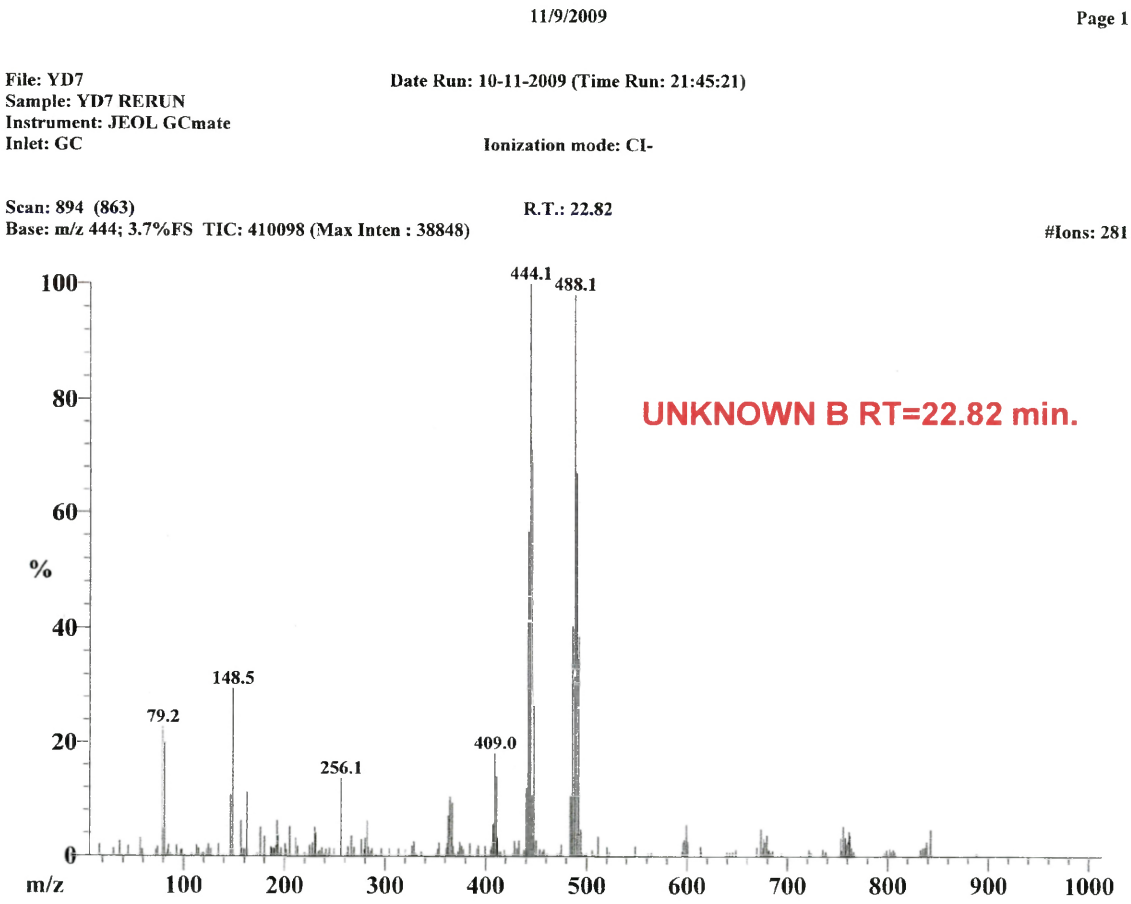
Figure R.12: Dibenzofuran Molecular Structure



dibenzofuran

The detection of this brominated furan raises questions. Of paramount importance is the origin of this compound. The dose verification samples were re-checked for this compound and other furans. None were found. The distribution samples were rechecked for this and other furans and some samples were found to contain this compound. This compound was only detected in the dosed yolk samples and in none of the other matrices from the distribution study. More specifically, only three of the dosed yolk samples showed the presence of this compound (YD3, YD7, and YD8). However, this PBDF was only detected in all three replicates of YD7. Only one replicate for the samples YD3 and YD8 showed the presence of this compound.

Figure R.13: Spectrum for Unknown Compound B with Retention Time of 22.82



The replicates for sample YD7 showed similar patterns.

The molecular ion is 842, the other clusters are 757, 673 and 599.

It has been suggested that this is a diphenyl ether with not chlorine and bromine substitutions that is an impurity in Deca-BDE. There are no data showing that this compound was present in ANY of the dosing emulsions. Ion 488 was plotted for the extractions of all the dosing formulations to confirm whether or not this was an impurity found in the dose itself. None of the medium dose replicates had a peak for ion 488 at the 22.82 retention time. The original

BDE-spiking solution used to make the dosing emulsions also does not show the presence of this compound. None of the Low Dose, High Dose, or Vehicle samples show this compound either. The yolk vehicle samples also show no sign of this compound. Again the 488 ion was plotted up for each sample.

This compound was found in all three replicates of every one of eight dosed yolk samples. The 444 ion was plotted up and all 24 samples had a peak at 22.82 minutes with the spectra shown in Figure R.13. The liver samples showed an identical pattern. All eight of the dosed liver samples had a peak at 22.82 with a spectra matching Figure HH. None of the liver samples in the vehicle treatment showed the presence of this compound.

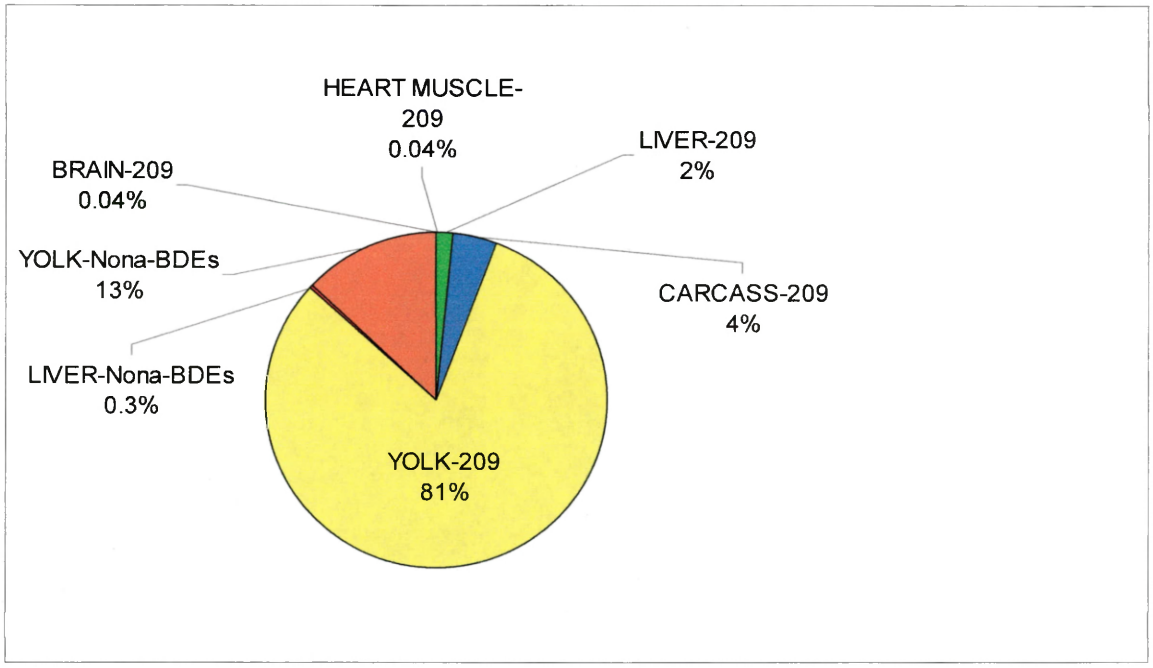
Examination of the standards used to make the calibration curve revealed the presence of this doubly halogenated compound. Therefore, the determination was made that the presence of this compound represents analytical cross contamination. Where this compound was detected the levels were extremely low; typically below the 1:10 signal to noise ratio required for quantitation.

R.7.5.6: Mass Balance Calculations

All the samples collected for the tissue distribution evaluation were derived from the Middle Dose treatment group. This treatment group was selected for analysis due to the survival rate throughout the experiment. Each egg was injected with 20 μg of BDE-209, so each embryo theoretically contained 20,000 ng of BDE-209. During collection the embryos were pooled into groups of four. Each pooled sample contains the select tissue from four individuals. Therefore, the dose must be summed over four embryos; 80 μg of BDE-209 is to be accounted for in the five tissue compartments of the embryo. This analysis first examines

the data from the entire distribution experiment using the mean concentration values from each of the tissue compartments. Follow-up analysis is done examining the individual sample pools and can be found in Appendix C.

Figure R.14: Distribution of BDE-209 in Yolk-Injected Chicken Embryos Using Mean Tissue Concentrations



The mean total carcass dry weight was 25.8 g. The mean concentration of BDE-209 detected in the dosed carcass tissue samples was 130 ng/g dw. This gives an average total amount of BDE-209 in the carcass tissues of 3400 ng, or 4.25 % of the total dose.

The mean total liver dry weight was 0.92 g. All the liver tissue was utilized during sample processing. The mean concentration of BDE-209 detected in the dosed liver samples was 1600 ng/g dw. This gives an average total amount of BDE-209 sequestered in the liver of 1470 ng, or about 2% of the total dose.

The mean total yolk dry weight was 19.33 g. The mean concentration of BDE-209 detected in the dose yolk samples was 3600 ng/g dw, yielding an average total amount of BDE-209 in the yolk compartment of 69,000 ng. This represents 87% of the total 80,000 ng delivered to each sample pool.

BDE-209 was not detected in any of the individual sample pools for the heart and brain tissues. The individual sample pools were combined and further concentrated. The pools of these small tissues exhibited nondetectable to low concentrations. The mean total dry weight of the hearts was 0.16 g. The mean concentration (of the reduced sample size n = 2) was 214 ng/g dw. This equates to less than 1% of the total dose for the sample pool.

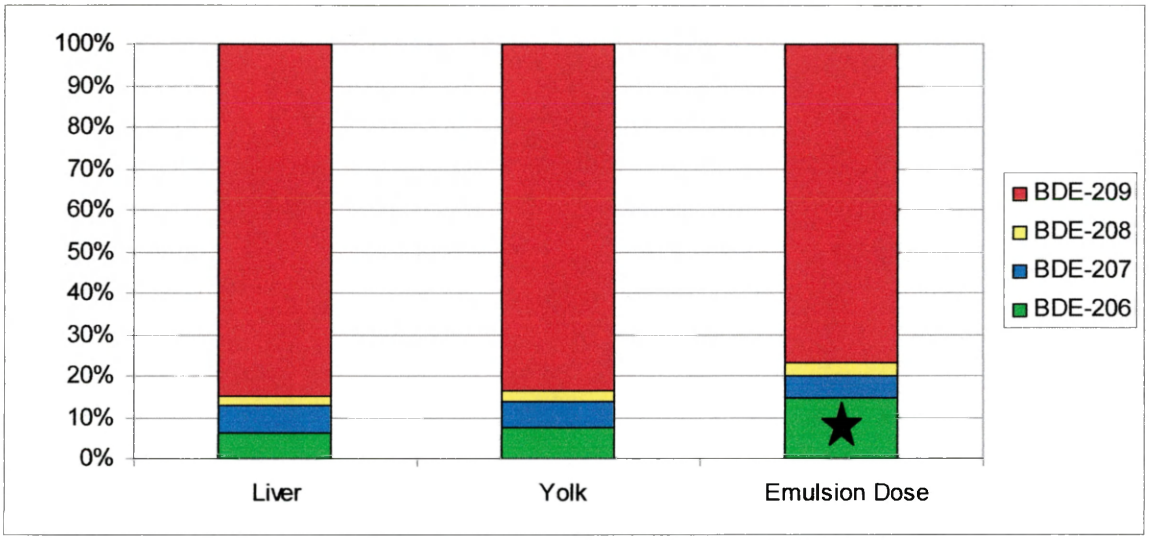
The mean total dry weight of the brain tissues was 0.54 g. BDE-209 was only detected in one (65 ng/g dw) of the two concentrated samples. However, this value is used due to the fact that the other sample only contained three rather than four of the pooled samples. This equates to less than 1% of the total dose.

Consideration of the nona-BDEs (BDE-207, 207 and 208) must also be taken into account. The mean total concentration of all three nona-BDEs in the yolk and liver tissues were quantified. In the dosed yolk samples, the mean total nona-BDE concentration was 580 ng/g dw. As the total mean yolk dry weight was 19.33 g, this represents a total nona-BDE contribution of 11,200 ng or 14% of the total administered dose. In the dosed liver samples, the total mean nona-BDE concentration was 260 ng/g dw. Again, the mean total liver weight was 0.92 g, yielding a total of 240 ng, or less than 1% of the total dose.

As the mean nona-BDE concentrations were elevated due to the presence of elevated levels in sample pool 7, a pool by pool mass balance analysis is indicated.

A one-way ANOVA and follow-up Tukey's test was run to determine if the congener composition of the original dose formulation, the yolk and liver compartments were statistically equivalent. At $\alpha = 0.05$, the only statistically significant difference was a higher BDE-206 percentage in the dose than the liver or yolks of the bird.

Figure R.15: PBDE Congener Profile for the Dosed Yolk, Dosed Liver, and Emulsion Dose



★ Statistically significant at $\alpha = 0.05$.

DISCUSSION

D.1: Toxicity of BDE-209

Data on toxicity of Deca-BDE to avian species are scarce. The analysis conducted here used the nominal concentrations of BDE-209 from trials three and four of this study. The requirements for this type of survival analysis were met; at least one treatment group resulted in a partial kill of the exposed population and there was a clear dose-response relationship. In this case, comparison of the Pearson Chi-Square values revealed that the Gompertz distribution was the best model, with a calculated LD50 value of 44µg/egg (740 ng/g ww). Selection of the most appropriate, and therefore accurate model, allows for a more accurate elucidation of the LD5 and LD10 values.

The Median Lethal Dose (LD50) is a basic metric used in risk assessment and toxicology. It identifies the dose of a given chemical at which half of the exposed study population dies. As such, it is useful for comparing the relative toxicities of chemicals. The chemical 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most toxic compounds in existence. As such, it is often used as the metric against which other toxins are measured against. In terms of Toxic Equivalency Factors (TEF) TCDD has the highest possible score of 1. The LD50 value reported for yolk injection of TCDD to chicken embryos is 122 pg/g egg (Henshel et al., 1997). This value is about 6000-times smaller than the LD50 value calculated in this study. Therefore, BDE-209 is about 6000-times less toxic to developing

chicken embryos than TCDD. This gives BDE-209 a relative potency of 0.000165 when compared to TCDD.

In birds, there are limited data available on the toxicity of PBDEs. Air-cell injection of the Penta-BDE mixtures resulted in decreased pipping and hatching success in American kestrels but not in chickens or mallards (Mckernan et al., 2009). Based on this dose and absorption rate of the dose from the aircell into the embryo, a Lowest Observed Effect Level (LOEL) of 1800 ng/g ww was determined. This is higher than the LD50 calculated in this study by a factor of 2.43.

In rats, a No Observed Effect Level (NOEL) for Deca-BDE of 8 mg/kg body weight per day in a 30 day dosing trial was determined by Norris et al. (1975). This was a traditional feeding study where Deca-BDE was added to food using a solvent carrier. The NOEL is a more sensitive endpoint than the LD50. It is the lowest treatment dose that elicited no statistically significant effect in a study where a dose-response effect was measured. For this study the NOEL ranged from 84 to 94 ng/g ww. The reported NOEL values from oral toxicity studies in mammals ranged from 100 mg/kg (Zhou et al. 2001) to 23,000 mg/kg (EPA 2008), with the majority of these studies not determining a LOEL (lowest observable effect level). These NOELs are much higher than the LD50 determined in the present study. The lack of studies reporting LD50 value for BDE-209 in mammals indicates that the compound is not very toxic to mammals in an acute exposure scenario. This is supported by the data showing the metabolic breakdown and excretion of BDE-209 in mammals. In birds, this mechanism results in the excretion of both the parent compound (BDE-209) and possible metabolic products into the egg. This results in a developmental exposure scenario.

A thorough toxicological review of BDE-209 was released by the US EPA in 2008 (EPA/635/R-07/008F). Minimal attention in this document was paid to acute lethal effects from BDE-209 exposure. In fact, the document fails to report any LD50 values for comparison. This is largely due to the fact that BDE-209 is believed to be relatively non-toxic. PBDEs emerged in the market place at a time when more toxic compounds (i.e. PCBs) were being removed due to toxic concerns. In the four decades of PBDE use, data have emerged that indicate that these compounds are not benign. Government restrictions are now in place limiting the use of PBDEs in a manner reminiscent of the treatment of PCBs. While, PBDEs are generally less toxic than their predecessors, the PCBs, they still behave as endocrine disrupting compounds (EDCs) in the environment. Therefore, it becomes crucial to examine the sublethal toxic effects of PBDEs and BDE-209.

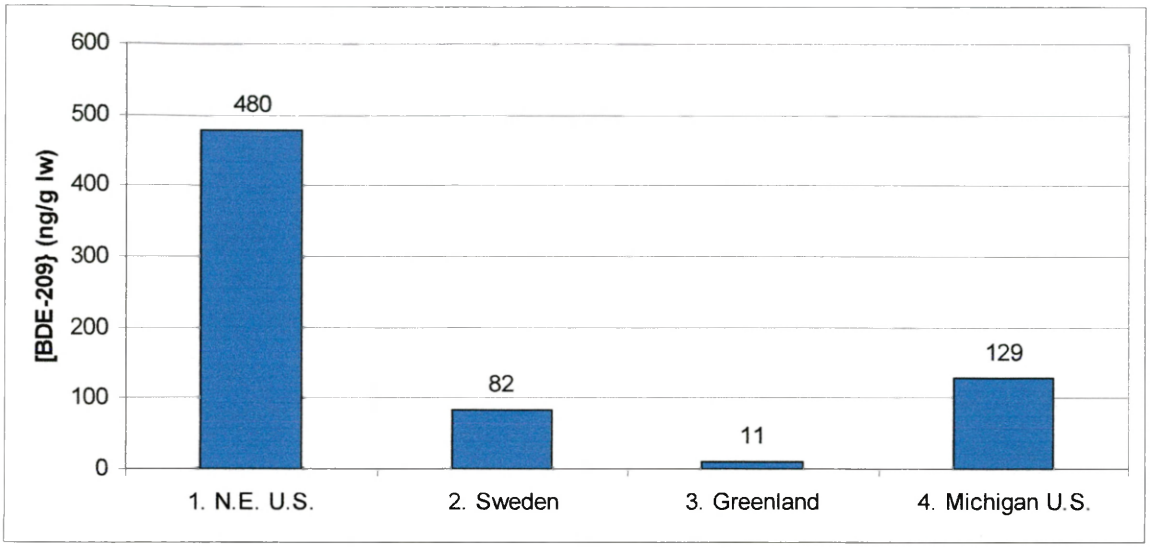
Sublethal effects have been well documented for BDE-209 in mammals. The 2008 EPA Document (EPA/635/R-07/008F) presents a thorough review of these studies. Reproductive and developmental toxicity values are much lower than the subchronic toxicity values discussed above for mammals. Neurobehavioral effects relating to chlolinergic system have been reported (Viberg et al. 2007). The LOEL values for these types of effects range from 6.7 mg/kg (Viberg et al.2007) to 20.1 mg/kg (Viberg et al. 2003) for the single dose studies. The LOELs from the multi-dose studies range from 6.1 mg/kg/day (Rice et al. 2007) to 500 mg/kg/day (Tseng et al. 2007). The LOEL for the present study was the middle dose of 20 μg/egg, which equates to 335 ng/g ww to 376 ng/g ww. These LOELs are an order of magnitude lower than the lowest values reported in mammals.

In a feeding study of American kestrels exposed to DE-71 and α-hexabromocyclododecane (HBCD) (Fernie et al., 2009) egg shell thickness declined with

increasing concentrations of PBDEs and HBCD. This effect was not correlated with levels of BDE-183 and -209. The potential for metabolic breakdown of these larger congeners to the more potent BDEs exists in the adult bird. Fernie et al. go on to postulate that PBDE and HBCD related egg shell thinning is already having an observable effect on brood size in Europe and the decline of the North American kestrel population.

Evaluating this toxicity value within the context of BDE-209 levels detected in bird eggs is important. The LD50 values from this study equated to concentrations ranging from 740 to 803 ng/g ww . Figure D.1 explores published BDE-209 levels in avian eggs. The levels of BDE-209 in avian eggs from around the world are all an order of magnitude less than the LD50 of 7000 ng/g lw calculated for this study. Conversion of the NOEL and LOEL to a lipid weight basis result in a range of 837-1340 ng/g lw (NOEL) and 538-3350 ng/g lw. In examining the potential toxic impact of these BDE-209 levels on real world bird populations, different lethal dose metrics may be examined. Again the accuracy of these numbers decreases as we move away from the middle (LD50) of the CDF. The LD10 calculated by the Gompertz model in this study was 21.8 μg/egg, the LD5 was 16.7 μg/egg. These values correspond to a range of 3000 to 3500 ng/g lw for the LD10 value and 2650 to 2800 ng/g lw for the LD5 metric.

Figure D.1: Levels of BDE-209 in wild bird eggs from various locations around the world



1. Gauthier et al. (2008) herring gulls 2. Lindberg et al. (2004) peregrine falcon 3. Vorkamp et al. (2005) peregrine falcon 4. Chen et al. (2008) peregrine falcon

The LD10 and LD5 values are still an order of magnitude higher than the BDE-209 levels detected in wild bird eggs. (See Figure D.1) The conclusion of this analysis is that BDE-209 levels in wild bird populations are lower than the threshold values for acute toxicity. It must be noted, that as the true mechanism of BDE-209 toxicity is not known in avian embryos, the potential for chronic effects still exists. Note that uncertainty factors applied are typically a factor of 10. Also that, in the present study, exposure was terminated before assimilation of the yolk sac and that the 80% of the BDE-209 remained in the yolk. Additional work is indicated to examine the distribution of this BDE-209 between waste and yet unutilized energy reserves and the potential impact of assimilation of the BDE-209 associated with the latter.

In an effort to explore sublethal endpoints in the present study, the embryos from the middle dose (20 μg/egg) and the Vehicle treatment groups were necropsied and the weights of

five tissues determined. Exposure to BDE-209 through yolk injection caused no significant change in brain weights, yolk weights, liver weights, heart weights, or total body weight. The tissue weight data were then transformed into a biological index value that expresses an organ weight as a percentage of total body weight. Again a one-way ANOVA with follow up Tukey's test was run. No statistically significant differences were detected between the tissue weight values or indices of the dosed and vehicle control injected embryos. However, the presence of edema and death in many of the embryos at the time of sampling precluded the use of tissue weight data in the analysis.

D.2: Dose Verification

Qualitative and quantitative dose validation is an important step in toxicological studies. The results of the dose verification indicated that the nominal doses were accurate enough to be used in the survival analysis and calculation of the LD50. The confirmation of the High Dose was the most consistent. The mean recovery was 91% with a standard deviation of 6%. The concentration of the High Dose emulsion was 800 μg BDE-209 per ml of emulsion. This High Dose emulsion was used in both trials three and four. In trial 3, 100 μl or 80 μg BDE-209 per egg of the High Dose emulsion was administered to the BDE-209 treatment group. In trial four, only 50 μl or 40 μg BDE-209 per egg of the High Dose emulsion was administered to the High Dose treatment group. In the survival analysis, the data from 5 treatments were used to create the CDF; the 80, 40, 20, 5 and the 0 μg BDE-209 per egg (vehicle treatment). Therefore, the confirmation of the High Dose at 91% recovery supports the accuracy of the dosages administered to both the 80 μg BDE-209 per egg treatment group and the 40 μg BDE-209 per egg treatment group.

Impurities within the technical BDE-209 used to make the emulsions must also be considered. In Figure R.1 the contributions of the BDE-206 and -207 are illustrated (determined from the Medium Dose Emulsion). When the concentrations of these two nona-BDE congeners are summed with the BDE-209 concentrations, the recoveries for the Medium Dose Emulsion are improved. The mean recovery for the Medium Dose Emulsion, accounting for the nona-BDEs, was 84.7%.

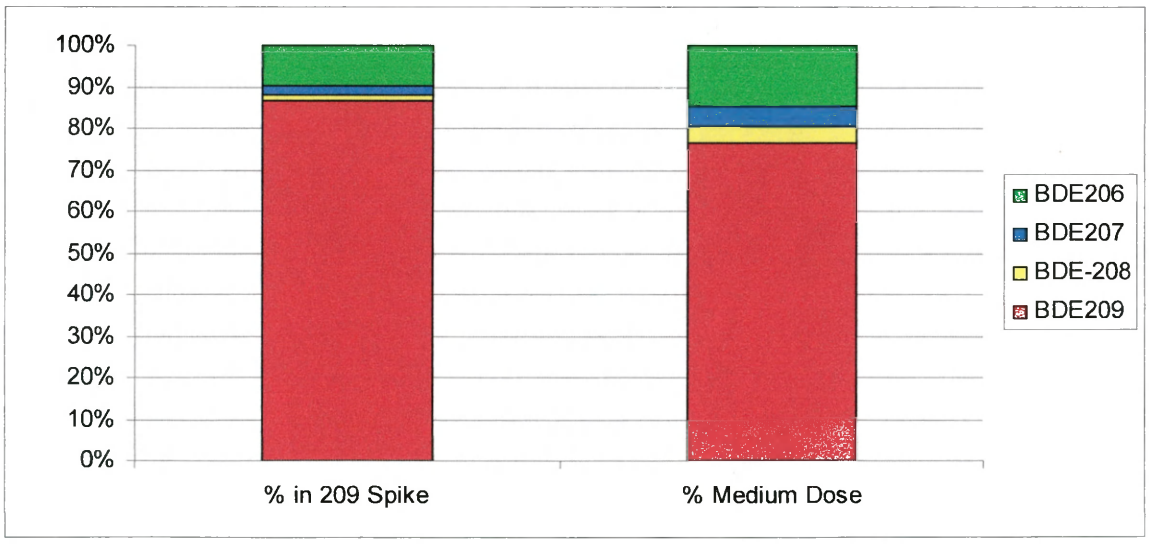
The possibility of non-PBDE impurities must also be addressed. BDE-209 is known to undergo photolytic debromination and furan formation (Hagberg et al. 2006, Kajiwara et al. 2008). Furans are highly toxic compounds. No furans were detected in the extracts of the dosing solution. Careful screening of the dose extracts was made for the Octa-PBDF that was identified in some of the dosed yolk samples. Again no furans were detected in any of the dosing solutions. This is likely due to the fact that BDE-209 solutions and emulsions were ALWAYS stored in amber glassware to prevent photolytic degradation that would confound the results of this study.

Even with the nona-BDEs taken into account, the recoveries for the Low Dose Emulsion were below 70%. While, this is less than ideal, it is not enough reason to abandon the nominal concentrations for the survival analysis. The analysis of the Emulsion Vehicle itself revealed no contamination of BDE-209. Therefore, accurate (80% or greater) confirmation of four of five doses administered in this study validate the use of nominal concentrations in the survival analysis.

The formulation of the emulsion vehicle required a highly concentrated BDE-209 spiking standard. BDE-209 is not very soluble in most solvents. Analytical standards for BDE-209 are not available for over 50 μg/ml. A concentration of 4 mg/ml BDE-209 was

desired to spike the emulsion formula. This standard was made by purchasing neat BDE-209 (Wellington Labs, Guelph, Ontario, Canada). The neat BDE-209 was dissolved in a solvent mixture of diethyl ether and toluene (1:1 v/v). To accomplish this, a probe sonicator was used for 30 minutes. The spiking solution showed 87% of the total PBDEs was BDE-209. The Middle Dose extraction has only 76% of the total PBDEs as BDE-209 (See Figure D.2). There are two possibilities that need to be addressed. First, did debromination of BDE-209 occur during the sonication and dissolution step? Second, did debromination of BDE-209 occur during the extraction step of the middle dose analysis?

Figure D.2: PBDE Percent Composition of the BDE-209 Spiking Solution and Middle Dose Extraction



The first question poses an interesting issue. We know that BDE-209 is photolytically labile; particularly so, when dissolved in solvent. The sonication/dissolution step was done in amber glassware. This reduces the likelihood of the photolysis pathway. The only other

mechanism would have to involve molecular excitement from the sonicator. While not impossible, this is thought to be unlikely.

The second question poses the possibility that debromination occurred during the dose extraction process. This is more likely. The emulsified dose was extracted in a separatory funnel after dilution. The funnel was not made of amber glass. Therefore, attempts were made to shield the contents from light. During the shaking and settling steps, the funnel was wrapped in aluminum foil. Unfortunately, the foil had to removed for the collection steps, in order to visualize the layers. This may have provided an opportunity for photolysis.

D.3: BDE-209 Egg Distribution and Biotransformation

D.3.1: Methodology

Chemical analysis of BDE-209 in biological tissues can be difficult due to its high molecular weight, high boiling point and potential to thermally degrade in the GC when subjected to high temperature. In a GC/MS, coincident biogenic compounds can distort chromatography and interfere with signal generation. These issues were observed in the course of analyses of some yolk samples in this study. The yolk of a developing chicken embryo acts as both a nutritional supply and waste collection compartment, containing lipids for consumption and metabolic byproducts, respectively. The analytical procedure was designed to remove water (freeze drier), extract lipophilic constituents (ASE) and remove extraneous materials from the analytes (SEC and silica SPE steps). Certain tissues, especially yolk, liver, and carcasses, contained greater amounts of extractable lipids. In effect, the high lipid content of both the yolk and carcass samples limited the amount of sample that could be analyzed at once, i.e. 1 and 1.5 g dry weight, respectively.

The presence of co-extracted biogenic compounds in the samples appeared to interfere with the BDE-209 signals in multiple spike recovery tests. Using the original extract purification method (LaGuardia et al. 2006a) apparent 2- fold increases in BDE209 was observed, for the yolk and carcass samples, respectively, data not shown. In addition, biogenic materials co-extracted from these matrices also accumulated on the GC column and impacted separation performance. For these reasons, further clean up of the sample extracts was required.

Alternate cleanup methods focusing on the final SPE step were investigated. The approach was to employ a weaker elution solvent regime to minimize carry-over of co-extracted biogenics. During the course of this work an interesting finding was made. BDE-209 was observed to be the first of the PBDE congeners to elute off the silica gel columns used. The initial expectation was that the less brominated congeners would elute earlier and BDE-209 would be the last of the PBDEs removed from the column. The opposite trend was observed. Grimvall and Ostman (1994) also observed this phenomenon. Bromine atoms are larger than chlorine atoms. Bromines are halogens like chlorines, however their placement in the periodic table makes them less electronegative than chlorines.

D.3.2: Tissue Distribution of BDE-209

Once an appropriate method of sample clean up was identified, the analysis of the samples en mass could begin. The results indicated that BDE-209 was mobilized from the yolk, into the heart, brain, liver, and remaining carcass of the developing chicken embryo. These findings are consistent with those from Van Den Steen et al. (2008). What remains unclear from the tissue distribution data is the true absorption rate of the compound into the

embryo. The fact that 80% of the total administered dose was found in the yolk clouds the issue. It is clear that some of the BDE-209 injected into the yolk was mobilized through the embryonic vascular system. It is not clear how much of the dose traveled through the embryo. The chicken yolk-sac acts as both a food source and a waste receptacle throughout incubation. Therefore, there is no way to confirm if the BDE-209 detected in the yolk-sac traveled through the developing embryo and was excreted back into the yolk-sac as waste, or if it is simply the unabsorbed remaining dose.

In mammals, neurological toxicity at low BDE-209 concentrations has been reported. Viberg et al have published multiple studies showing that neonatal exposure to BDE-209 may impact spontaneous behavior and habitutation. Viberg et al (2003) revealed that BDE-209 can pass the blood brain barrier in neonatal mice. Viberg et al (2007) also showed that rats exposed to BDE-209 during the neonatal brain growth spurt show altered responses to nicotine in adulthood. Viberg et al (2008, 2009) later examined the impact of neonatal BDE-209 exposure to neural protein concentrations in rats. The findings of these studies indicate that in mammals BDE-209 enters the brain and alters protein concentrations in ways that can cause permanent neurological changes if the exposure occurs during the brain growth spurt (BGS).

A major objective of this study was to determine the tissue distribution of BDE-209 within the avian embryo. Previous work has indicated that BDE209 in rats does not distribute strictly according to the concentration of lipid with tissues in rats (Huwe et al 2008). They reported preferential accumulation of BDE209 in liver and muscle. BDE-209 was detected in one of the composite pooled brain samples. This indicates that BDE-209 does pass the blood-brain barrier in the chicken embryo. Therefore, the potential for neurological effects similar

to those reported in mammals (Viberg et al., 2008) exists in birds. It is also noteworthy, that while BDE-209 was detected in very low concentrations (<BQL) in the brains of the dosed birds. The BDE-209 detected in the brains of the dosed embryos accounts for only 0.04% of the total administered dose. The brain samples were all less than 1 gram. This led to a rather high quantitation limit of 2000 ng/g lw.

BDE-209 was also detected (Below the Quantitation Limit or BQL) in the composite dosed heart muscle samples. This is consistent with the idea that BDE-209 was mobilized from the yolk compartment through the vascular system of the developing embryo. The literature seems to suggest that BDE-209 is mobilized in the blood (Huwe et al. 2008) and is relatively high in avian muscle tissue. Liang et al. (2008) identified BDE-209 as the dominant PBDE congener in foraging chicken hens from an electronic waste recycling area in southern China. Liang et al. (2008) showed peak BDE-209 levels in muscle tissue of 17,977 ng/g lipid wt. The levels reported in the heart tissue were lower than skeletal muscle, with an average of 1135 ng/g lw. Like the brain samples, very little sample was available for analysis, only 0.1 g per pool of four. Therefore, these samples were reduced in volume and pooled together to lower the detection limits. Again, there was a trade off here between statistical power and detection limit, the reason the samples were analyzed individually first. Since BDE-209 was not detected, the detection limit must be lowered to confirm this finding. However, when pooling tissues and concentrating extracts, one may also encounter additional matrix interference issues in the GC/MS.

The fact that BDE-209 was detected in all of the dosed carcass samples indicated that it spread from the yolk into the embryo during development. The most logical mechanism for this movement was through the blood. Van den Steen et al. (2007) monitored BDE-209 in the

blood of adult starlings. The muscle tissue BDE-209 concentrations were two-fold the liver tissue BDE-209 concentrations in the Van den Steen study.

All of the liver samples from dosed eggs contained relatively high concentrations of BDE-209. The average concentration of 3600 ng/g lw represents about 7% of the total 20,000 ng/egg dose. The presence of high levels of BDE-209 in the liver was anticipated. Many researchers have reported BDE-209 concentrations in liver tissue of wild or free-ranging birds. Jaspers et al. (2006) observed 68 ng/g lw of BDE-209 in the liver tissue of barn owls from Belguim. Luo et al. (2009) reported a mean BDE-209 concentration of 18 ng/g lw in liver tissue from chickens in South China. In a similar study, Liang et al. (2008) found 495 ng/g lw in the liver of chickens at an electronics recycling site in South China. Chen et al. 2007 detected the highest concentrations of BDE-209 in livers, of Chinese birds of prey, a mean value of 12,200 ng/g lw..

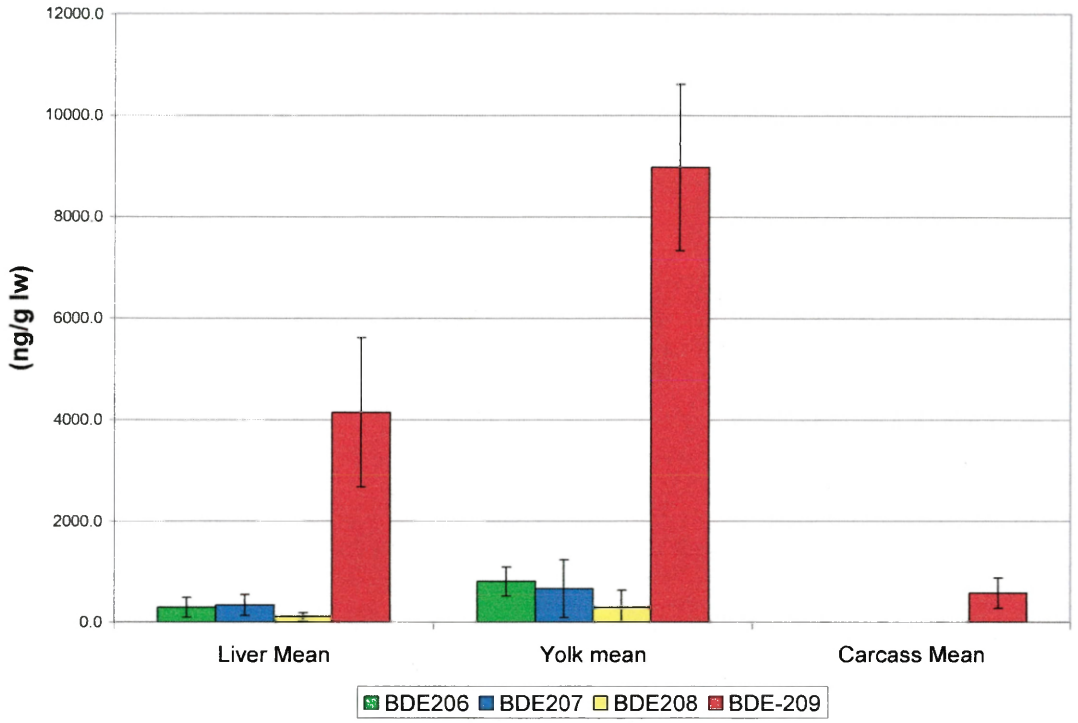
D.2.3: Other PBDEs detected

The three possible nona-BDEs (BDE-206, 207 and 208) were detected in all of the dosed yolk and liver samples. Nona-BDEs were not detected in any of the carcass, brain or heart muscle samples. The sum of the average concentrations for each of the individual nona-BDEs in the dosed liver samples was 741 ng/g lw. If this is added to the average liver BDE-209 concentration of 3600 ng/g lw the total would be 4340 ng/g lw. The average total lipid weight for the pooled liver samples was 0.40g. This amounts to 1736 ng BDE-209 in the liver compartment instead of 1440 ng. The change is relatively insignificant in calculating the percentage of the total dose, the new value is 8.7% versus the old value of 7.

The sum of the average concentrations for each of the individual nona-BDEs in the dosed yolk samples was 1800 ng/g lw. If this is added to the average liver BDE-209 concentration of 9000 ng/g lw the total would be 10,800 ng/g lw. The average total lipid weight for the pooled liver samples was 7g. This amounts to 75,000 ng BDE-209 in the yolk compartment.

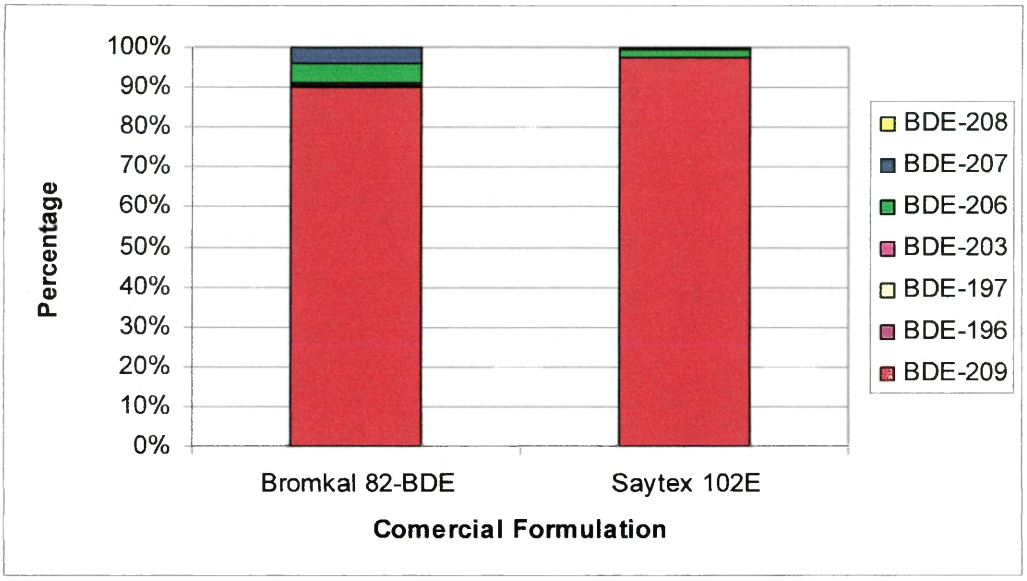
In viewing the yolk samples individually, some interesting patterns emerge. As all of the samples represent a pool of four tissues, individual analysis can not be conducted. The pool becomes the experimental unit and may mask some of the variation that occurred within the individual embryos. Figure D.2 shows the BDE-209, -208, -207, and -206 trends in the yolk samples. Sample YD7 clearly exhibited the lowest levels of BDE-209 and the highest levels of all three nona-BDEs. This is the type of pattern one would expect to see if metabolic debromination was occurring in some embryos.

Figure D.3: Mean Deca- and Nona-BDE concentrations in pooled liver, yolk, and carcass samples from BDE-209 dosed eggs



In the yolk samples it is clear that BDE-206 is the dominant non-BDE congener. This is not the case in the liver samples. In the liver samples BDE-207 is the dominant nona-BDE congener. It is unclear if this is due to metabolic formation of BDE-207 in the liver. No octa- or hepta-BDEs or lower (other than BDE-166) were detected in any of the samples. A comparison of the congener profiles of the samples from this study and those of the available Deca-BDE commercial formulations is required. Figure D.4 shows the congener profiles of the two most common Deca-BDE products (LaGuardia et al., 2006).

Figure D.4: Congener profiles of two commercial Deca-BDE formulations



Data from La Guardia et al. (2006)

In both commercial Deca-BDE formulations presented in Figure D.4 BDE-206 is the dominant nona-BDE congener. The Bromkal 82-BDE product contains trace amounts of octa-BDEs (<0.5%). The Saytex 102E product does not contain octa-BDE impurities. No octa-BDEs were detected in any of the samples from this study. If the octa-BDEs were present then they were always BQL.

D.2.4: Other brominated compounds detected

The detection of an Octa-PBDF in three of the yolks samples raised some questions. There are several possibilities to explain the presence of this compound therein. One possibility is that an impurity existed within the BDE-209. Indeed, Hanari et al (2006) reported octabromodibenzofuran to be a substantial impurity of the commercial decaBDE product DE-83. It was the dominant brominated furan observed, present at up to 48 ng/g.

Alternatively, the PBDF may have formed through photolytic breakdown of BDE-209 during the analytical process. However, except for the lipid determination and final solvent volume reduction steps, the samples were always stored in amber glassware to prevent photolytic degradation of BDE-209. The literature indicates that PBDFs may be created by photolysis of BDE-209 (Hagberg et al. 2006; Kajiwara et al. 2008). Both of these studies identified numerous PBDFs in light-xposed BDE-209 samples. For example, Hagberg et al. (2006) identified 27 different PBDFs breakdown products. While they did not monitor the hepta- and octa- brominated PBDFs, Kajiwara et al. (2008) did in an experiment investigating the photolysis of BDE-209 in plastics. However, again multiple PBDF congeners were reported. The majority of these were the lesser brominated tri through hexa- PBDFs. This information leads this author to hypothesize that this single, fully-brominated PBDF was not formed through photolytic degradation as more samples would have likely been affected and additional PBDF congeners formed.

The third possibility for the occurrence of this furan was laboratory contamination. This can not be completely ruled out, but it also seems unlikely. Contamination of only five samples out of a total of 96 indicates that the contamination was not systematic in the analytical method. Furthermore, this compound is not kept in the laboratory inventory and is not used in commercial products. So, the contamination would have had to some from a light exposed solution of BDE-209 or other source. Quality control measures should have prevented this. Again, had the contamination come from a photolytically degraded BDE-209 solution, it is likely that other furans would also be present.

A fourth possibility is that this furan was formed inside the embryo. Formation of this compound would require the loss of two bromines from the para- positions on each ring and

the subsequent formation of the C-C bond between the two rings. The theorized mechanism for debromination in birds is the reductive debromination by 5'-MA (deiodinase), preferentially at the meta (5) position (Valverde et al. 1993). Therefore, the loss of two bromines from the para- positions can not be explained by this specific mechanism. The potential exists for an alternate mechanism within the embryo to explain this observation. However, the details of that mechanism are not understood.

CONCLUSION:

The findings of these studies indicate that BDE-209 is toxic to chicken embryos when injected into the yolk, exhibiting an LD50 of 740 ng/g over a 20 day exposure via yolk injection. This is roughly 6000 times less toxic than TCDD to embryonic chickens. The distribution analysis of the five compartments (yolk, liver, brain, heart, and carcass) is consistent with published literature indicating that BDE-209 is transported by the blood throughout the embryo. No definitive evidence of metabolic debromination by chicken embryos was seen. Both tissue extracts and the dosing emulsion exhibited low levels of nona-BDE congeners. Highest BDE-209 concentrations were in the yolk at the conclusion of the study, day 20 (just prior to pipping). The yolk compartment acted as both a source (from the initial injection) and a likely sink (recipient of waste products) for BDE-209 and contributed to this result. A more complex distribution model (i.e. with more compartmentalization) is recommended for future similar work. In addition, I would recommend that the embryos be allowed to hatch, absorb all of their yolk and the BDE-209 in the yolk. Then sacrificed at 3-4 days of age to determine how much of the administered dose actually ended up in tissues and how much was excreted and /or metabolized. This author believes a microsomal assay similar to that from Stapleton et al. (2006) could be used to address the question of metabolic debromination of BDE-209 by avian embryos.

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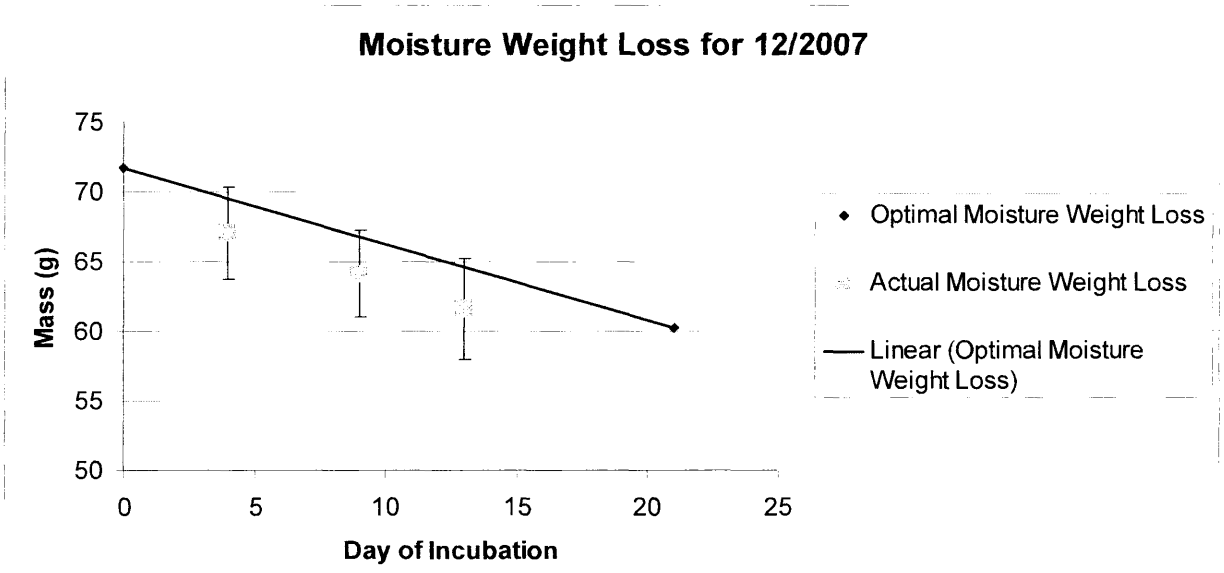
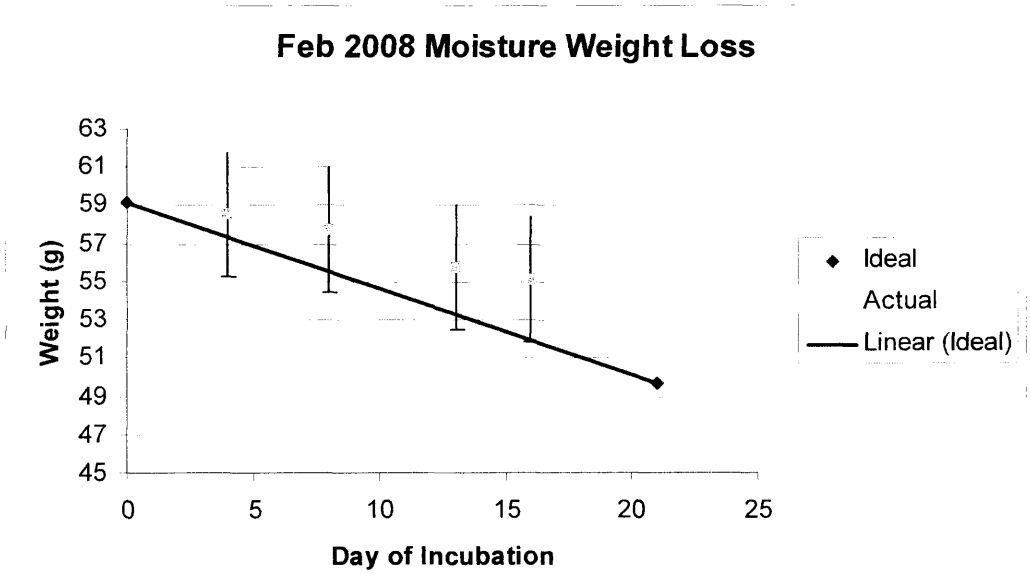
Appendix A: Moisture Weight Loss throughout Egg Incubations

Figure A.1: Moisture Weight Loss for Trial 1



Error bars show one standard deviation in each direction.

Figure A.2: Moisture Weight Loss Trial 2



Error bars show standard deviation.

Figure A.3: Moisture Weight Loss in Trial 3 Non-Injected Eggs

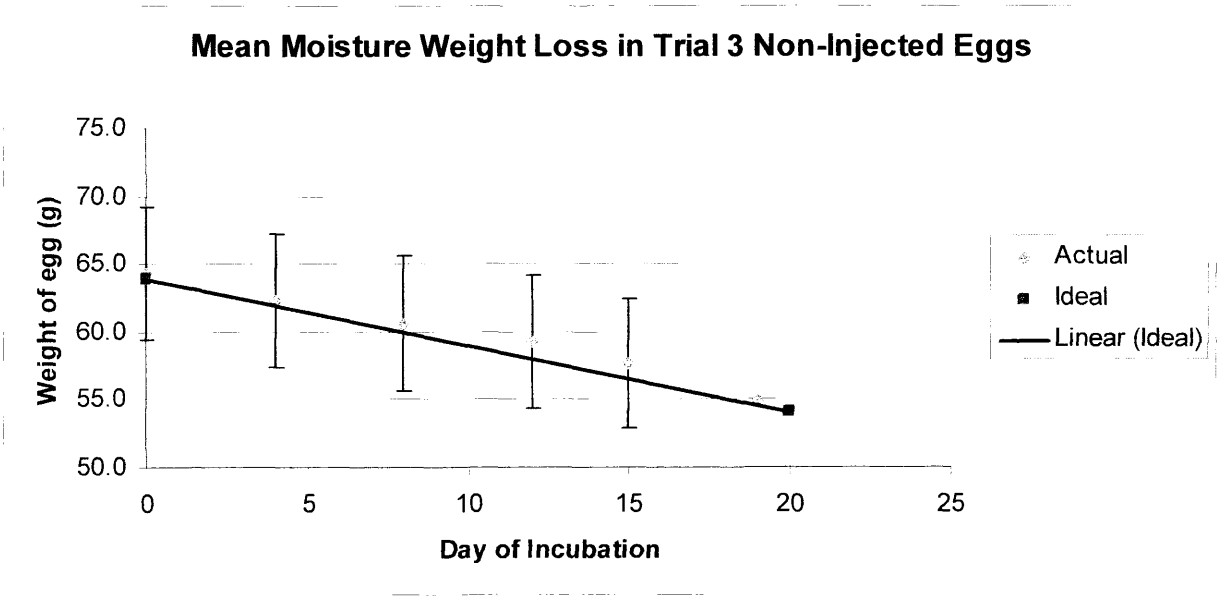


Figure A.4: Moisture Weight Loss in Trial 3 Vehicle Injected Eggs

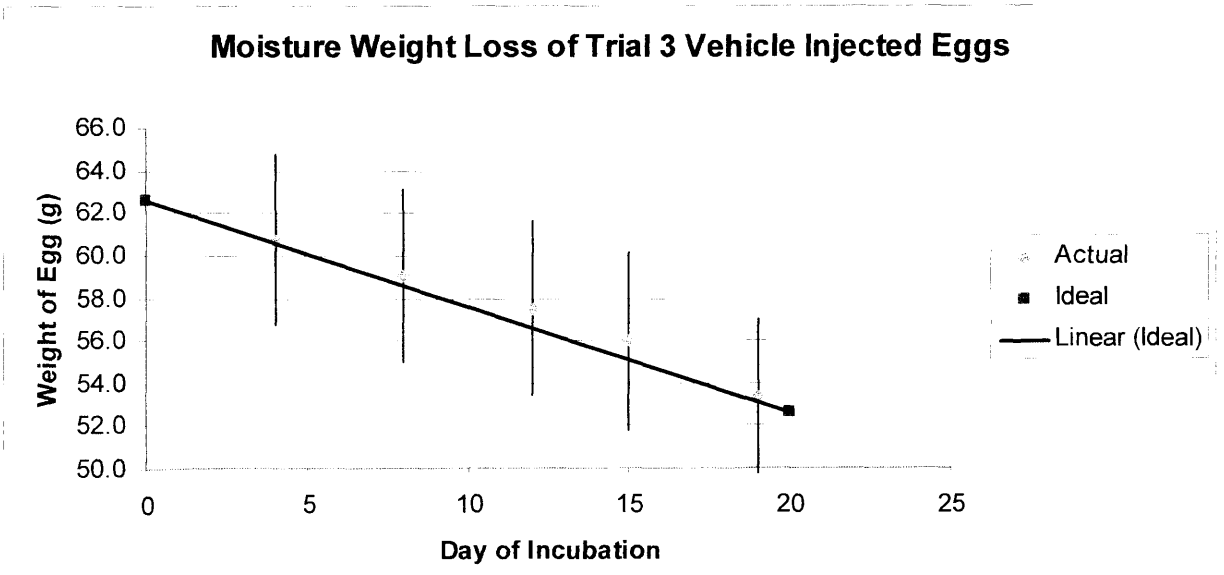
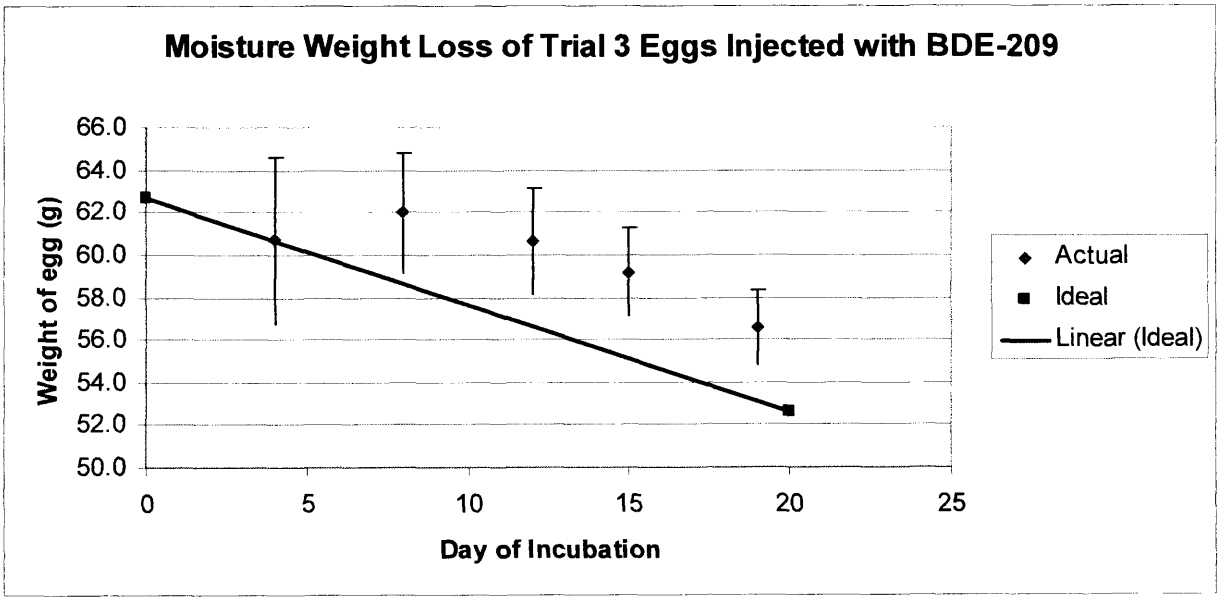


Figure A.5: Moisture Weight Loss in Trial 3 BDE-209 Injected Eggs



Appendix B: Conversion of Tissue Compartment Dry Weights to Wet Weights

Figure B.1 : Dry Weight Versus Wet Weights of Pooled Brain Samples

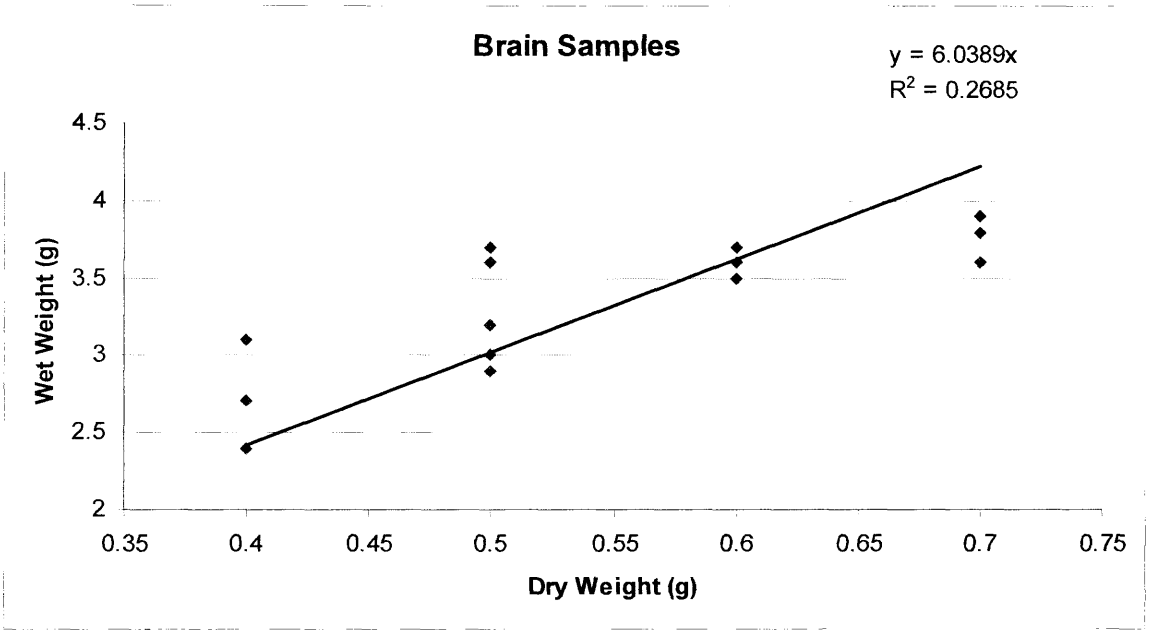


Figure B.2: Dry Weight vs. Wet Weight for Pooled Carcass Samples

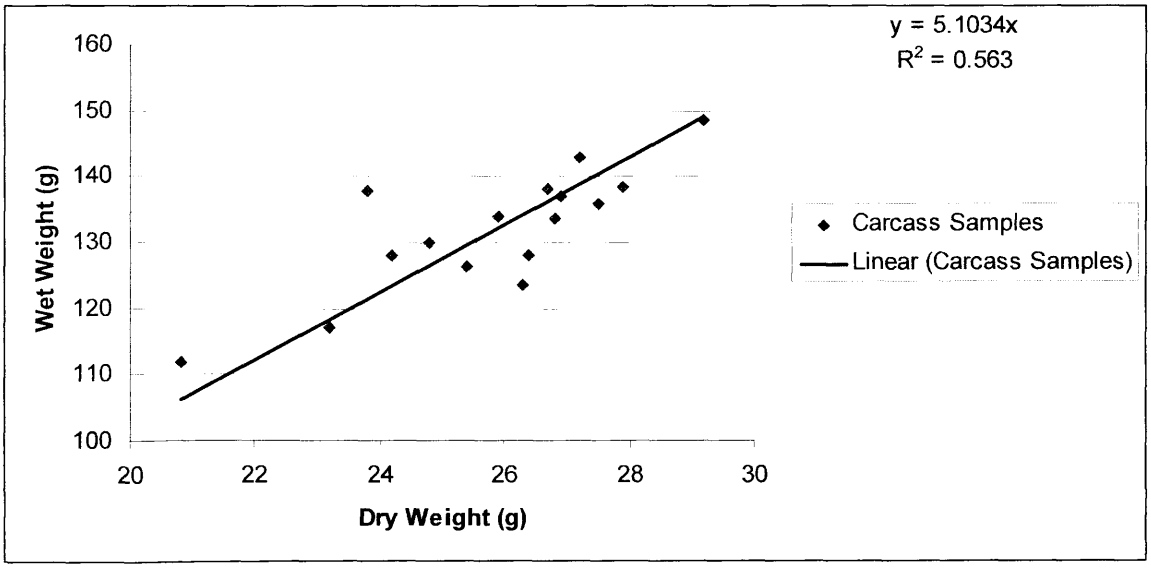


Figure B.3 Comparison of sample dry weights to wet weights.

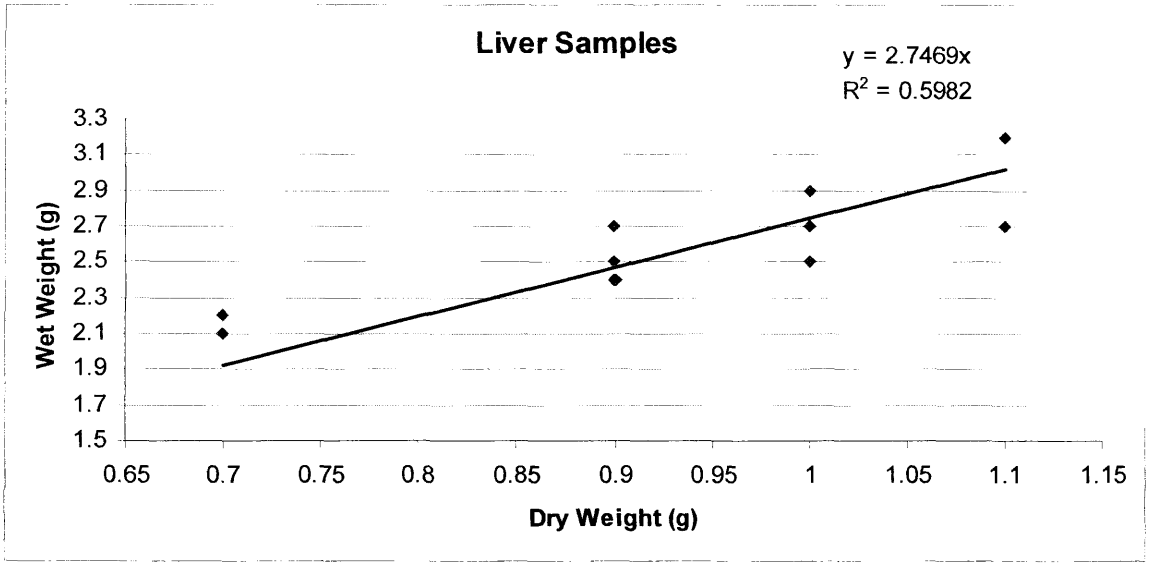


Figure B.4: Heart Muscle Samples Wet Weight vs. Dry Weight

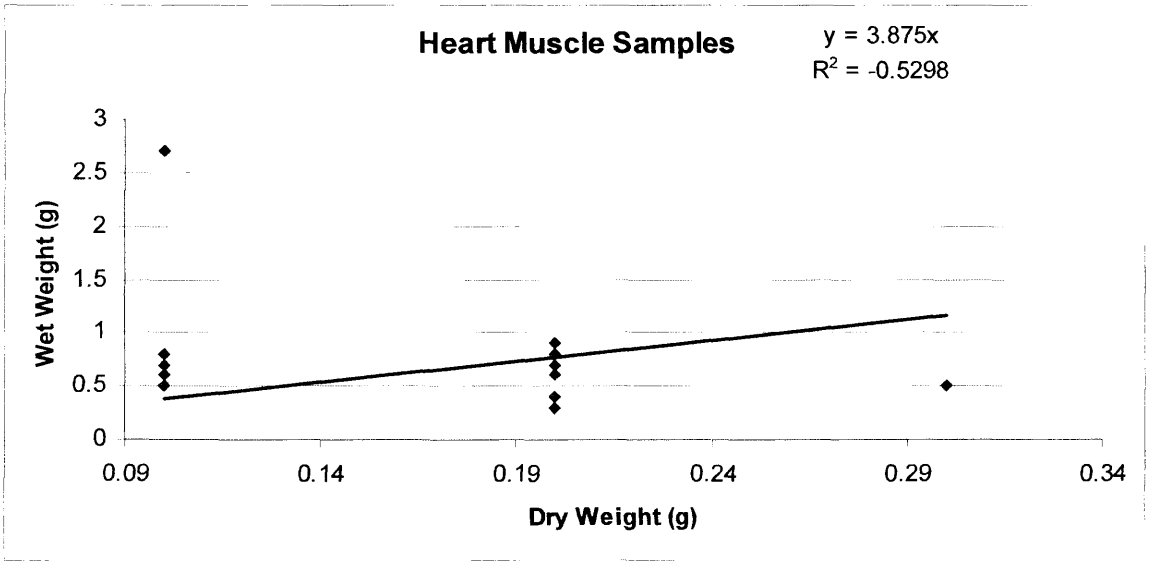
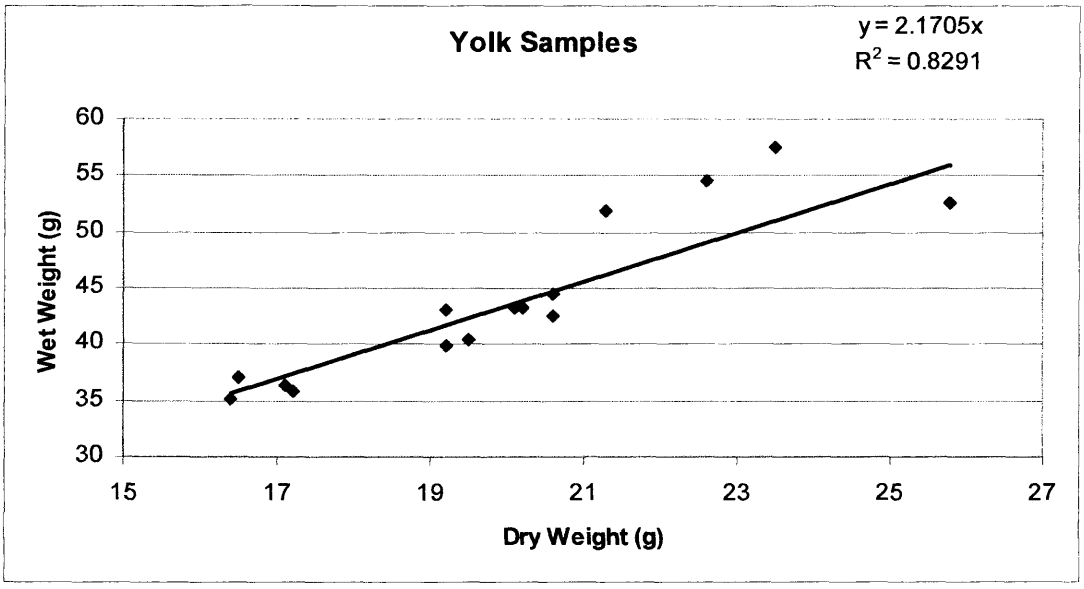


Figure B.5: Yolk Samples Wet Weight vs. Dry Weight


Yolk Samples
y = 2.1705x
R2 = 0.8291
Wet Weight (g)
60
55
50
45
40
35
30
15
17
19
21
23
25
27
Dry Weight (g)

Appendix C: Mass Balance on an Individual Sample Basis

Sample Pool 1

Sample LD1 was lost during sample processing. Therefore, the mean liver concentration is used in these calculations. The data are presented in Table R.23. A total of 48,800 ng of BDE-209 is accounted for in the form of BDE-209. A total of 8200 ng of BDE-209 can be accounted for in the form of the three Nona-BDEs. This leads to total detection of roughly 71% of the total administered dose to Sample Pool 1.

Table C.1: Sample Pool 1

	BDE209 (ng/g dw)	Total BDE-209(ng) in pooled Tissue Sample	Sum Nona-BDEs (ng/g dw)	Total Sum Nona-BDEs (ng) in pooled tissue sample
BD1	0	0	0	0
MD1	0	0	0	0
LD1	1800	1800	260	260
CD1	116	3000	0	0
YD1	220	44000	390	7900
	SUM	**48800**	**SUM**	**8160**

Sample Pool 2

Table C.2: Sample Pool 2

	BDE209 (ng/g dw)	Total BDE-209(ng) in pooled Tissue Sample	Sum Nona-BDEs (ng/g dw)	Total Sum Nona-BDEs (ng) in pooled tissue sample
BD2	0	0	0	0
MD2	0	0	0	0
LD2	880	790	130	120
CD2	105	2900	0	0
YD2	3500	60000	470	8000
	SUM	**63690**	**SUM**	**8120**

Sample Pool 3

Table C.3: Sample Pool 3

	BDE209 (ng/g dw)	Total BDE-209(ng) in pooled Tissue Sample	Sum Nona-BDEs (ng/g dw)	Total Sum Nona-BDEs (ng) in pooled tissue sample
BD3	0	0	0	0
MD3	0	0	0	0
LD3	1000	110	230	250
CD3	110	2940	0	0
YD3	3100	68000	660	13600
	SUM	**71050**	**SUM**	**13850**

Sample Pool 4

Table C.4: Sample Pool 4

	BDE209 (ng/g dw)	Total BDE-209(ng) in pooled Tissue Sample	Sum Nona-BDEs (ng/g dw)	Total Sum Nona-BDEs (ng) in pooled tissue sample
BD4	0	0	0	0
MD4	0	0	0	0
LD4	1850	1850	370	370
CD4	80	2200	0	0
YD4	3900	74880	410	7900
	SUM	**78930**	**SUM**	**8270**

Sample Pool 5

Table A.5: Sample Pool 5

	BDE209 (ng/g dw)	Total BDE-209(ng) in pooled Tissue Sample	Sum Nona-BDEs (ng/g dw)	Total Sum Nona-BDEs (ng) in pooled tissue sample
BD5	0	0	0	0
MD5	0	0	0	0
LD5	1170	1170	220	220
CD5	70	1780	0	0
YD5	3700	74400	370	7400
	SUM	**77350**	**SUM**	**7620**

Sample Pool 6

Table C.6: Sample Pool 6

	BDE209 (ng/g dw)	Total BDE-209(ng) in pooled Tissue Sample	Sum Nona-BDEs (ng/g dw)	Total Sum Nona-BDEs (ng) in pooled tissue sample
BD6	0	0	0	0
MD6	0	0	0	0
LD6	2600	2340	390	351
CD6	140	3800	0	0
YD6	4100	70500	370	6400
	SUM	**76640**	**SUM**	**6751**

Sample Pool 7

Table C.7: Sample Pool 7

	BDE209 (ng/g dw)	Total BDE-209(ng) in pooled Tissue Sample	Sum Nona-BDEs (ng/g dw)	Total Sum Nona-BDEs (ng) in pooled tissue sample
BD7	0	0	0	0
MD7	0	0	0	0
LD7	1840	1660	430	390
CD7	97	2400	0	0
YD7	4200	80600	1400	26880
	SUM	**84660**	**SUM**	**27270**

Sample Pool 8

Table C.8: Sample Pool 8

	BDE209 (ng/g dw)	Total BDE-209(ng) in pooled Tissue Sample	Sum Nona-BDEs (ng/g dw)	Total Sum Nona-BDEs (ng) in pooled tissue sample
BD8	0	0	0	0
MD8	0	0	0	0
LD8	1900	1710	210	190
CD8	320	8400	0	0
YD8	3860	63700	380	6270
	SUM	**73810**	**SUM**	**6460**

VITA

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