December 14, 2011

Apparent Tolerance of Common Tern (*Sterna hirundo*) Embryos to a Pentabrominated Diphenyl Ether Mixture (DE-71)

Barnett A. Rattner, Rebecca S. Lazarus, Gary H. Heinz, Natalie K. Karouna-Renier, and Robert C. Hale

USGS-Patuxent Wildlife Research Center, Beltsville, Maryland

Virginia Institute of Marine Sciences, Gloucester Point, Virginia

Executive Summary

Recent polybrominated diphenyl ether (PBDE) embryotoxicity studies with American kestrels (*Falco sparverius*) suggest the lowest-observed-adverse-effect-level (LOAEL) for pipping and hatching success to be 1.8 µg penta-BDE/g egg on a wet weight (ww) basis (~32 µg/g lipid on a dry weight basis). Concentrations of total PBDEs in Forster's tern (*Sterna forsteri*) eggs from the San Francisco Bay range up to 63 µg/g lipid on a dry weight basis, and thus exceed this LOAEL. As a surrogate for west coast terns, 60 recently laid common tern (*Sterna hirundo*) eggs were collected from Poplar Island, Maryland. Six eggs were chemically analyzed and all were found to contain low levels of organochlorine pesticides (<0.08 µg/g ww), total polychlorinated biphenyls (PCBs) (<0.45 µg/g ww), and total PBDEs (<0.052 µg/g ww), indicating that eggs from this mid-Chesapeake Bay location could be used for studying the commercial PBDE DE-71 formulation for embryotoxicity. Eggs were artificially incubated, and on day 4 of development either corn oil vehicle (n=19, controls) or DE-71 at doses of 0.2 µg/g (n=12), 2 µg/g (n=12), or 20 µg/g (n=19) was injected into the air cell at constant volume (0.5 µl/g egg). There were no significant effects of DE-71 on survival to 90% of incubation (DE-71: 63.6 - 90.9% vs. 87.5% for controls), to pipping (63.6 - 81.8% vs. 81.2% for controls), or to hatching (63.6 -75.0% vs. 81.2%). However, DE-71 treated eggs hatched 0.44 days later than vehicle controls (p=0.0137). No gross deformities were observed in tern embryos that failed to hatch or in tern hatchlings. Crown-rump length and the weight of the hatchling, yolk sac, liver, bursa of Fabricus, thyroids, and organ to body weight ratios were not affected by DE-71, although spleen weight and spleen to body weight ratio of the 2 µg/g group were greater than controls (p=0.05 and 0.03). Lengths of the tibiotarsus, metatarsus, femur, humerus, and ulna in cleared skeletal preparations did not differ among groups. Histopathological examinations of

liver, kidney, spleen, bursa of Fabricius, and thyroid were unremarkable, and oxidative stress measurements (total and protein bound sulfhydryl, oxidized and reduced glutathione, thiobarbituric acid reactive substances) in hatchling terns were not affected by DE-71. As a positive control, a study was conducted in which American kestrel eggs either received corn oil vehicle (n=36) or 20 µg DE-71 per gram egg (n=34) by injection into the air cell. Although DE-71 did not impair pipping and hatching success of kestrels, it did result in a delay in hatch, shorter humerus length, and lower total thyroid and total thyroid to body weight ratio. Concentrations of oxidized glutathione, reduced glutathione and thiobarbituric acid reactive substances were all greater in DE-71 treated kestrels compared to controls. Our findings suggest common tern embryos, and perhaps other tern species, are no more sensitive (and are probably less sensitive) to PBDEs than are American kestrel embryos.

Introduction

San Francisco Bay provides critical habitat for millions of birds, contains three Important Birds Areas, and has been designated as a Wetland of Hemispheric Importance (Chipley et al. 2003). Pesticides, industrial chemicals, metals and other anthropogenic compounds enter the Bay through many routes (runoff, sewage outfalls, atmospheric deposition), and constitute a potential threat to fish and wildlife resources. Since the 1970's, polybrominated diphenyl ethers (PBDEs) have been commonly used as additive flame retardants in polymers, textiles, and electronics. In 2004, the United States Environmental Protection Agency (US EPA) and various manufactures reached agreement to phase out the use of certain penta- and octa-BDE formulations because of concerns about their persistence, bioaccumulation, and toxicity to wildlife, humans and the environment. The deca-BDE formulation continues to be used in the United States, but upon the realization that it breaks down to more toxicologically problematic congeners, the largest commercial manufacturers voluntarily agreed to phase out its use by 2012 (US EPA 2011). A multi-stakeholder partnership between the US EPA and various interest groups is currently examining environmentally safe flame retardant substitutes for the deca-BDE formulation.

Some PBDE congeners readily bioaccumulate and biomagnify in food chains, and monitoring studies have demonstrated that concentrations increased in eggs of wild birds nesting in North America (e.g., herring gulls, *Larus argentatus*) between 1980 and 2000 (e.g., Great Lakes, Norstrom et al. 2002), and remained elevated thereafter (Gauthier et al. 2008). On a wet weight basis (ww), concentrations of total PBDEs in avian eggs ranged up to 1.40 µg/g ww in herring gulls from the Great Lakes (Norstrom et al. 2002), 1.88 µg/g ww in ospreys (*Pandion haliaetus*) from Willamette River in Oregon (Henny et al. 2009), and 6.60 µg/g ww in peregrine

falcons (*Falco peregrinus*) from the northeast US (Chen et al. 2008). Concentrations of total PBDEs in Forster's tern (*Sterna forsteri*) eggs from the San Francisco Bay range up to 63 µg/g lipid on a dry weight basis (She et al. 2008). Meta analysis of PBDE congener profiles in birds indicated distinctly different congener profiles between terrestrial and aquatic food webs (e.g., terrestrial webs exhibit lower biomagnification of BDE-47 compared to aquatic webs) (Chen and Hale 2010).

Studies in birds have examined sublethal biochemical, immunological, developmental and reproductive effects of environmentally relevant concentrations of PBDEs (see review by Chen and Hale 2010). For example, Fernie and co-workers (2005a, 2005b, 2006) injected American kestrel (*Falco sparverius*) eggs with 18.7 µg total PBDEs on day 19 of incubation (i.e., 1.43 µg/g egg), and then nestlings were orally gavaged daily with the same PBDE mixture at 15.6 ng/g body weight/day through day 29 post-hatch. Using this combined egg injection/dietary exposure regime, there was some evidence of increased growth (i.e., body weight, tarsometatarsus and feather length) (Fernie et al. 2006) and structural changes in immune organs (i.e., fewer germinal centers in spleen, reduced apoptosis in bursa, increased macrophages in thymus) (Fernie et al. 2005a) in kestrels administered PBDEs. As carcass concentrations of BDE-47 and BDE-183 increased, some alterations in immune function (i.e., greater phytohemagglutinin skin response and reduced antibody-mediated response) were detected (Fernie et al. 2005a). The PBDE mixture also evoked oxidative stress (i.e., marginal increases in GSSG:GSH ratio, oxidized glutathione, and in lipid peroxidation) in kestrel nestlings. Additionally, in nestlings (29 days old), plasma thyroxine (T4), plasma retinol and hepatic retinol were inversely related to carcass concentrations of BDE-47 and -99 (Fernie et al. 2005b). In another study by Fernie and coworkers (2008), reproductively active adult kestrels were fed

concentrations (0.3 µg/g diet or 1.6 µg/g diet) of a commercial penta-BDE formulation (DE-71) and changes in reproductive behavior were noted (e.g., fewer bonding behaviors, copulated less, and spent less time in nest box) (Fernie et al. 2008). Exposure of kestrels to DE-71 causes a delay in egg laying, smaller eggs, some evidence of eggshell thinning, and reduced fertility and reproductive success (Fernie et al. 2009).

Perhaps more germane to the interpretation of PBDE concentrations in bird eggs, chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*) and kestrel eggs were treated with 0.01 to 20 µg PBDE/g egg (McKernan et al. 2009, 2010). Decreased pipping and hatching success in kestrels were observed at nominal concentrations of 10 and 20 µg/g egg. Measurement of the quantity of the air cell-injected PBDE that actually entered the egg contents (yolk, albumen, extra embryonic membranes and embryonic tissues) indicated that the lowest-observed-adverse-effect level (LOAEL) on pipping and hatching success in kestrels was 1.8 µg/g egg ww (~32 µg/g lipid on dry weight basis). This threshold has been exceeded in some egg samples from free ranging birds (Chen et al. 2008, She et al. 2008, Henny et al. 2009). In these egg injection studies, sublethal effects (P450 induction, reduced bursal mass and follicle size) were also detected in chicken hatchlings. In contrast, mallard embryos seemed to be relatively insensitive to PBDEs.

It was recognized that similar data on the relative sensitivity of terns to PBDEs could greatly assist natural resource managers assessing the risk these chemicals pose to terns and other species of waterbirds that nest in San Francisco Bay. Thus, a study was undertaken in 2010 with the following objectives:

1. Determine embryonic survival, pipping and hatching success of common terns to air cell-administered polybrominated diphenyl ether (DE-71 formulation).

2. Examine embryos and hatching terns for evidence of sublethal effects including deformities, growth, hepatic, thyroid and immune organ histopathology, and biochemical effects.
3. Compare responses and the relative sensitivity of common terns to results found in other similarly tested avian species (chicken, mallard and kestrels).

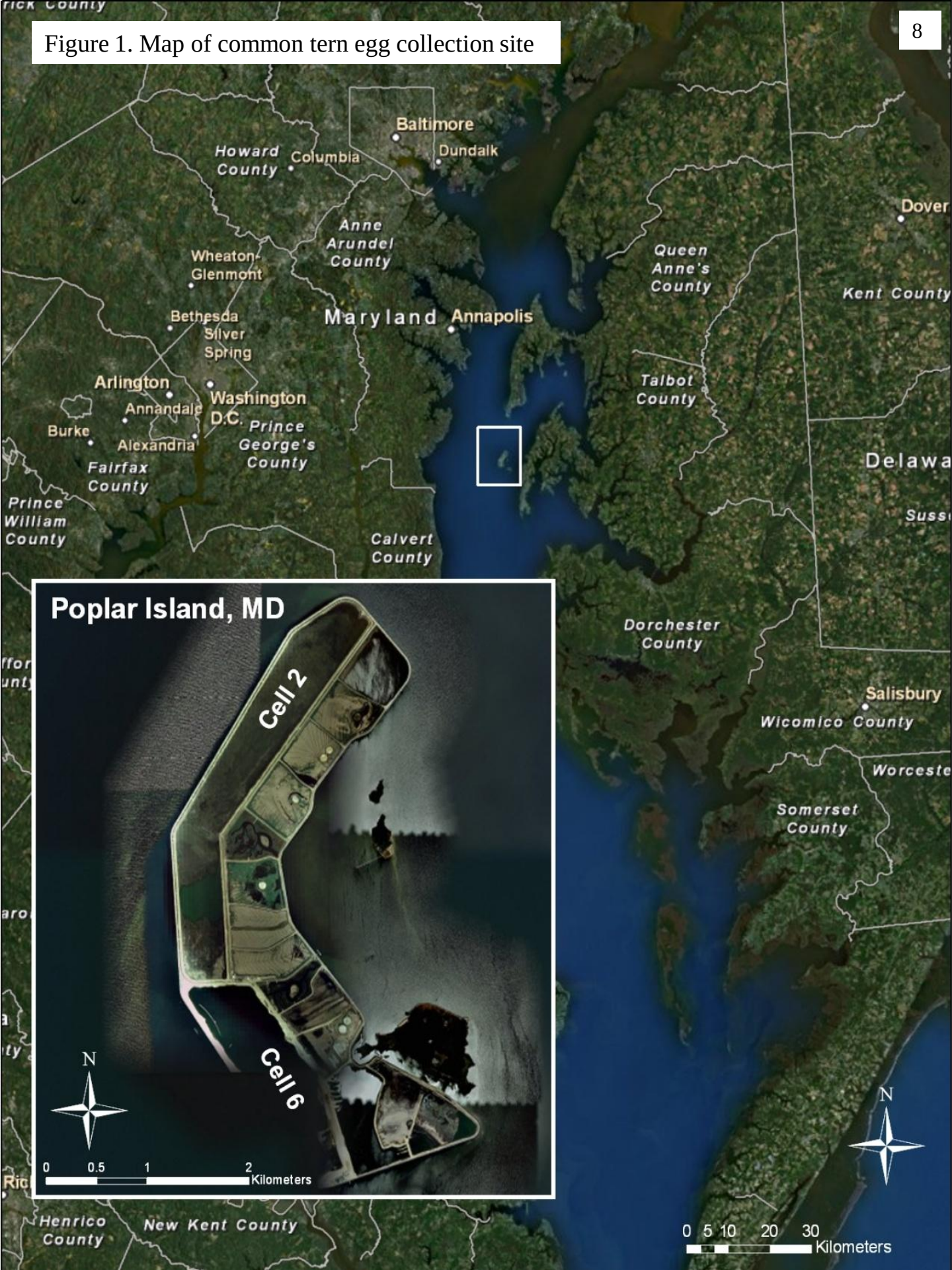
Materials and Methods

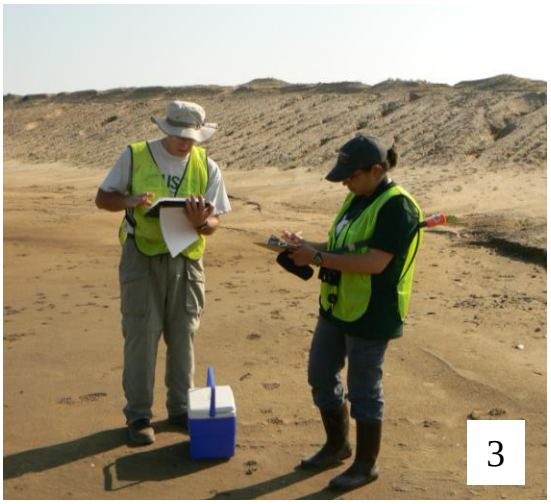
Egg collection

On May 25, 2010 a visit was made to Poplar Island in the Chesapeake Bay (Figure 1) of Maryland for the purpose of marking any common tern nests that contained only one egg; these single eggs were marked to enable identification of freshly laid second eggs during a subsequent visit (Figure 2). On May 27, a second visit was made, and 60 fresh common tern eggs were collected under appropriate state and federal collecting permits (Figure 3). To avoid collecting eggs that had already undergone some incubation, eggs from only two categories of nests were collected: eggs in single-egg unmarked nests and eggs that could be identified as the second egg in two-egg nests where the first egg had been marked in the earlier visit. A few of the eggs were floated (Hays and LeCroy 1971), and based on their flotation angle (close to horizontal) they were deemed as being relatively freshly laid (Figure 4). The eggs were transported back to the lab in egg cartons, weighed, numbered 1 through 60 (Figure 5), and left tilted on their side to sit overnight at room temperature (approximately 21°C).

Kestrel eggs were collected April 8-14, 2010 from a captive colony at the Patuxent Wildlife Research Center, Laurel, Maryland. The eggs were stored in a Kuhl® egg cooler (Kuhl Corporation, Flemington, NJ) at approximately 11-13°C and 70-76% relative humidity (RH) and

Figure 1. Map of common tern egg collection site





Figures 2-14: Pictures from common tern study. Figure 2. Common tern nest on Poplar Island, MD; Figure 3. Collecting common tern eggs; Figure 4. Floating eggs to determine incubation stage; Figure 5. Number identifying common tern eggs; Figure 6. Washing eggs with 1% Aplicare; Figure 7. Kuhl incubator used in study

were rotated 60° every hour. On April 15, the eggs were removed from the egg cooler to equilibrate to room temperature.

Incubation of eggs

Incubation of the common tern eggs began on May 28, 2010. The eggs were first washed in a 40°C solution of 1% Aplicare® (active ingredient: 10% povidone-iodine), followed by a rinse in 40°C clear tap water, and air dried (Figure 6). The dry eggs were placed on their sides, in a Kuhl® incubator and incubated at 37.5°C. The incubator automatically rotated the eggs approximately 180° in one direction and then, an hour later, 180° in the opposite direction (Figure 7). When the eggs of most species of birds are incubated by the parents they lose approximately 15% of their weight up to the time of pipping (Rahn and Ar 1975). Based on our own experiences with artificial incubation of wild bird eggs, we adjusted this number to 16% (Klimstra et al. 2009). The only way to achieve approximately 16% weight loss of individual eggs of a wild species of bird is to periodically weigh each egg (generally 3 day intervals) and plot its path toward the 16% endpoint. Periodic changes in RH must be made along the course of incubation to achieve this ideal weight loss. To achieve the desired 16% weight loss of each egg, we used three different incubators, each with a different setting for RH. Owing to changes in the RH and temperature in the incubator room, it was impossible to perfectly control the RH inside the three artificial incubators. Therefore, the settings (which were called low, medium, and high RH) actually fluctuated between approximately 14-27%, 24-37%, and 58-62% RH, respectively. Percent moisture loss by pipping was estimated as the [(fresh weight-weight at pipping)/fresh weight] x 100. Eggs were candled and weighed to the nearest 0.1 g (Acculab VI-400, Newton PA) every 2 to 4 days, and eggs were sometimes transferred from one incubator to another to

steer the moisture loss line for each egg toward 16%. In the later stages of incubation, we also used a viability detection instrument (Buddy®; Vetronic, Torquay, UK) which is a digital egg monitor that detects the heartbeat of the embryo (Figure 8). Any eggs determined to have died were removed from the incubator and opened for examination. On June 16, the eggs were transferred to a Kuhl® hatching unit set at 37.2°C and approximately 70% RH. Hatching success was recorded for each set of eggs. Once the first egg hatched on June 18, eggs were monitored carefully from 0700h-2345h from June 18-June 20 (until the last egg hatched). For birds that hatched between 2345-0700h, approximate hatch time was roughly estimated based on the appearance of the chick (i.e., wetness of down feathers).

Incubation of the kestrel eggs began on April 15, 2010. The eggs were weighed, labeled 1-70, and washed in the same manner as had been done with the common tern eggs. Kestrel eggs were incubated on their sides in a Kuhl® incubator set at 37.5°C, and were rotated approximately 180° every hour. As with the incubation of the common tern eggs, it was necessary to use three incubators with different RH in order to ensure each egg lost approximately 16% of its weight by the time of pipping. Eggs were shifted as necessary among incubators with low RH (13-33%), medium RH (28-37%), and high RH (60-73%). Eggs were candled and weighed every 2 to 4 days, and in the later stages of incubation the Buddy® also was used to determine if an embryo was alive or dead (Figure 8). Dead eggs were removed from the incubator and opened for examination. On May 9, the kestrel eggs were transferred to a Kuhl® hatching unit set at 37.2°C and approximately 70% RH. Hatching success was recorded for each set of eggs. After the first egg hatched on May 12, eggs were checked hourly from 0700-1800h. During daylight hours, hatching time was determined based on direct

Figures 8-13: Pictures from common tern study. Figure 8. Determining egg viability with Buddy; Figure 9. Injection of DE-71 into air cell; Figure 10. Pipping common tern egg; Figure 11. Common tern hatchling; Figure 12. Measuring crown-rump length of common tern hatchling; Figure 13 .Skeletal preparation of hatchling

observations. For birds that hatched between 1800-0700h, approximate hatch time was estimated based on the appearance of the chick (i.e., wetness of down feathers).

Dosing solutions

Neat DE-71 provided to Dr. Robert Hale by the Great Lakes Chemical Corporation (GLCC; Kalamazoo, MI) was used in this study as the source of the DE-71 GLCC formulation (LGC Promochem, Teddington, UK) employed in earlier work (McKernan et al. 2009, 2010) is no longer commercially available. Due to the viscous nature of the material, the container of neat DE-71 was warmed for 5 minutes with a stream of 75°C air emitted from a Varitemp heat gun (model VT-750, Master Appliance Corp., Racine, WI). Approximately 200 mg was transferred to a 5 ml volumetric flask to which 50 µl acetone was added and then brought to the mark with corn oil (Sigma-Aldrich, St. Louis, MO). The flask was covered with aluminum foil and mixed with a stir bar on a magnetic hot plate for 1 hour at 55°C. From this stock solution, various quantities of DE-71 were further diluted in volumetric flasks containing vehicle (corn oil plus acetone at 1% by total volume). Nominal concentrations were 0.2, 2 and 20 µg DE-71 per 0.5 µl solution.

Dosing of eggs

Common tern eggs were injected after approximately 101 hours (4 days and 5 hours) of incubation. Eggs were removed from the incubator and set in a vertical position. The blunt (cap) end of each egg was cleaned with an alcohol swab, and a 1/8–inch hole was drilled through the cap. The oil solutions were heated to 40°C, and each egg was injected into the air cell with vehicle or DE-71 solution at a volume of 0.5 µl per gram of egg. Randomly selected eggs were

injected with vehicle (controls, n=19), 0.2 µg DE-71 per gram of egg (n=12), 2 µg DE-71 per gram of egg (n=12), or 20 µg DE-71 per gram of egg (n=17) (Figure 9).

The hole in the cap was sealed with a vinyl acetate adhesive and the eggs were held vertical at room temperature for 30 min to allow the corn oil to spread out over the inner shell membrane. At the end of this period, the eggs were returned to the incubator.

Kestrel eggs were injected after approximately 121 hours (5 days and 1 hour) of incubation, using the same protocol as was used with the common tern eggs. Randomly selected eggs were injected with vehicle (controls, n=36) or 20 µg DE-71 per gram of egg (n=34).

Necropsy of eggs that failed to hatch and hatchlings

Embryo viability during incubation was monitored at 3-4 d intervals by candling or with a viability detection instrument. Embryos that died during development or failed to pip were removed from the eggshell and evaluated for stage of development and presence of abnormalities. Survival through 90% of the incubation period, incidence of pipping (Figure 10), and hatching success were determined.

Six common tern and three American kestrel eggs that were vehicle injected controls (infertile or died early in incubation) were prepared for chemical analysis. To adjust for moisture loss and facilitate determination of fresh weight contaminant concentrations, a small hole was drilled in the egg shell at the top of the air cell and distilled water was injected into the air cell to return the volume to the initial fresh egg volume (Heinz et al. 2009a). The weight of the egg containing the added water was determined and egg contents were removed and transferred to a chemically-clean glass jar and weighed. Embryos were examined to determine if eggs were infertile or died early in development. Egg contents were stored at -80°C and saved for chemical

analyses of a suite of organochlorine pesticides, total polychlorinated biphenyls (PCBs) and PBDEs to determine background contamination.

Day-old hatchlings were examined for evidence of edema and teratogenicity (e.g., eye, foot, bill deformities) (Figure 11). Each hatchling was weighed, crown-rump length determined (Figure 12), and then sacrificed via decapitation. The yolk sac, liver (minus the gall bladder), spleen, bursa of Fabricius, and thyroids were removed and weighed. Body, yolk sac and liver were weighed to the nearest mg (Ohaus Precision Advanced GT410, Parsippany, NJ) and other organs were weighed to the nearest 0.1 mg (Sartorious R200D, Goettingen, Germany).

A small piece of the liver, and the bursa, spleen and thyroids was fixed in formalin for histopathogical examination. A small piece of liver was placed in RNAlater, and the remainder of the liver was divided between two cryovials, snap frozen in liquid nitrogen, and stored at -80°C for oxidative stress assays.

Skeletal preparations and histopathology

After sacrifice and sample collection, the remaining carcass of each kestrel and common tern hatchling was labeled and stored in 70% ethanol. Carcasses were cleared, feathers removed, and skeletons were stained by the method of Karnofsky (1965) (Figure 13). Crown-rump, tibiotarsus, metatarsus, femur, humerus and ulna lengths were measured to the nearest millimeter with electronic digital calipers using a lighted examination surface. Measurements were taken on the left side of the embryo. Visual examinations were conducted as well to identify any deformities.

Formalin-fixed liver, kidney, spleen, bursa of Fabricius and thyroids were embedded in paraffin, sectioned at 5µm, 2 step sections mounted on slides, and then stained with hematoxlyin

and eosin (American HistoLabs, Gaithersburg, MD, USA). A preliminary evaluation of tissue sections by light microscopy was undertaken by one of the principal investigators. A subset of 27 common tern samples (control, n=5; 0.2 µg/g egg, n=5; 2 µg/g egg, n=5; 20 µg/g egg, n=12) was further examined for unusual findings, abnormalities, and severity grading of findings (not remarkable, minimal, mild, moderate, and moderately severe) by a Board Certified Veterinary Pathologist (Dr. Jeffrey C. Wolf, EPL, Inc, Sterling, VA).

Oxidative stress assays in liver tissue of hatchlings

Liver tissue was analyzed for four measures of oxidative stress: total sulfhydryl (TSH), total glutathione (TotGSH), reduced glutathione (GSH), and thiobarbituric acid reactive substances (TBARS). Protein bound sulfhydryl (PBSH; TSH minus GSH), oxidized glutathione (GSSG = [TotGSH-GSH]/2), and the ratio of GSSG to GSH (GSSG:GSH) were calculated using the measured endpoints.

Frozen liver samples were thawed on ice and homogenized on ice in 2X PBS (pH 7.4; Fisher BioReagents, Waltham, MA, USA) at 200 µg/µl. The homogenate was centrifuged at 10,000 x g for 10 min at 4°C and aliquots of the supernatant were transferred to 0.2 ml tubes and frozen at -80°C until analysis. For GSH, GSSG, and TotGSH analysis, liver supernatant was thawed and diluted to 12.5 µg/µl in 1X PBS and analyzed using the DetectX® Glutathione Fluorescent Detection Kit (Arbor Assays, Ann Arbor, MI, USA) following the manufacturer's protocol. TBARS levels were determined using the QuantiChrom™ TBARS Assay Kit (Bioassay Systems, Hayward, CA, USA). For determination of TBARS (indicative of lipid peroxidation), sample supernatant was thawed on ice and diluted to 100 µg/µl in 1X PBS. To 300 µl of sample, we added 50 µl of 48% TCA and then followed the manufacturer's

instructions. Total sulfhydryl was determined in thawed supernatant that had been diluted to 5 or 6.3 µg/µl in 1X PBS using the Measure-iT ™ Thiol Assay Kit (Invitrogen- Molecular Probes, Inc., Eugene, OR, USA) following the manufacturer's instructions. All assays were analyzed using a Fluostar Omega microplate reader (BMG Labtech Inc., Cary, NC, USA). The limit of quantitation (LOQ) was determined as the mean of +10× the standard deviation of sample blanks (average of replicates across three separate assays). The LOQs were 0.06 µM, 11.3 µM, 0.20 µM, and 0.26 µM for TBARS, TSH, GSH, and TotGSH, respectively. Each plate included blanks and three pooled reference samples from kestrel, common terns, and tree swallows (*Tachycineta bicolor*), respectively, to account for plate-to-plate variability. Each sample was analyzed in duplicate from which a mean was calculated and used for analysis. Intrassay variability was maintained below 15%. The coefficient of variation (% CV) of duplicates (mean ± standard deviation) was 2.6 ± 2.6%, 4.4 ± 3.7%, 2.9 ± 2.5%, and 2.1 ± 1.5 % for TBARS, TSH, GSH, and TotGSH, respectively. Samples for which % CV of the duplicates exceeded 15% were reanalyzed.

Chemical analysis of dosing solutions and eggs

Sample Preparation

Dosing solutions containing DE-71 were diluted in hexane and an internal standard (p-terphenyl) was added. These solutions were diluted and analyzed by GC/MS, as described below. Tern and kestrel egg analysis was conducted based on the methods of Chen et al. (2008, 2009). Eggs were freeze-dried, and a surrogate standard (200 ng of 2,3,4,4′, 5,6-hexabromodiphenyl ether, BDE-166; Cambridge Isotope Laboratories, Inc., Andover, MA) was added to each sample. Spiked egg samples were subjected to accelerated solvent extraction

(Dionex ASE 200, Sunnyvale, CA), and extracts were purified by size exclusion chromatography (SEC, Envirosep-ABC®, 350 x 21.1 mm. column; Phenomenex, Torrance, CA). Each post-SEC extract was reduced in volume, added to the top of a solid phase 2-g silica glass extraction column (Isolute, International Sorbent Tech., Hengoed Mid Glamorgan, UK) and eluted with 3.5 ml hexane (fraction one), followed by 6.5 ml of 60:40 hexane/dichloromethane (DCM) and then 8 ml DCM (fraction two). The second fraction, containing the compounds of interest, was reduced in volume and solvent exchanged to hexane. Decachlorodiphenyl ether (DCDE; 100 ng) was then added as the internal standard (Ultra Scientific, North Kingstown, RI) and the extracts analyzed for 57 PBDE congeners (single or co-eluting pairs). Identification and quantitation were done by gas chromatography mass spectrometry using selective ion monitoring (GC-MS/SIM).

PBDEs using GC-MS/SIM

Diluted dosing solutions and purified egg extracts were analyzed by GC (6890N, Agilent Tech., Palo Alto, CA) with MS detection (JMS-GC Mate II, JEOL, Peabody, MA). Sample aliquots (1 μl) were introduced into the GC split/splitless injector, equipped with a glass liner (1 mm, i.d.), and separated on a 15-m DB-5HT (0.25 mm i.d., 0.1 μm, J & W Scientific, Agilent Tech.) column. The injector temperature was 300^{o}C and initial carrier gas (helium) head pressure was 50 psi. Four minutes after sample injection, the split vent was opened and pressure reduced to 15.2 psi (flow 1.2 ml/min). Column flow rate was kept constant (1.2 ml/min, temperature compensated) throughout the remaining portion of the analysis. Initial column oven temperature was 90^{o}C, held for 4 min, then increased to 150^{o}C at 30^{o}C/min, then 10^{o}C/min to

300°C, and held for 20 min. Column oven temperature was then increased to 350°C at 30°C/min and held at 350°C for 5 min. Total run time was 47.7 min.

The MS was operated in the electron capture negative ionization (ECNI) mode (methane reagent gas (99.99%) using SIM. Ion source temperature was 200°C, transfer line was maintained at 300°C. Quantitation ions for PBDEs were *m/z* 79 ($[^{79}Br]^-$), 81($[^{81}Br]^-$). Confirmation ions were 486 and 488 *m/z* for decabromodiphenyl ether (BDE-209). A five-point calibration curve, $r^2 > 0.995$, was constructed from the analysis of calibration standards (Wellington Laboratories, Inc., Ontario, Canada). The quantitation limit for the 57 PBDE congeners in eggs was 0.2 ng/g ww and 5 ng/ml for analytes in the corn oil solutions.

Organochlorines

Purified egg extracts were analyzed using a multipoint calibration curve generated using the responses of the authentic standards of the targeted analytes (viz., 41 organochlorine pesticides and metabolites, octachlorodibenzodioxin, octachlorodibenzofuran, and 68 individual or co-eluting PCB congeners) versus that of the internal standard. A Varian Saturn 4D ion trap GC-MS operating in the electron ionization mode was used to analyze the samples. A DB-5 60mX0.33 idX.25um film capillary column (J & W Scientific, Folsom, CA) was installed in the GC. The GC was programmed the following: 75°C 1 min hold, 75°C to 350°C at 4°C/min, 350°C hold 15.25 min. The MS ion mass range collected was 100-650 *m/z*. Individual analytes were quantified using selected ions. The quantitation limit for analytes in eggs was 0.2 ng/g ww.

Statistical Analysis

Data were analyzed using SAS ® (SAS Institute 9.2). For the common terns, survival to 90% of incubation, to pipping, and to hatching were analyzed using Chi-Square Analysis with

the Bonferroni Correction to account for multiple treatment groups. Additionally, incidence of histopathological abnormalities between groups was analyzed by Chi-Square test. For both experiments, residuals were tested for normality (Shapiro Wilk test statistic (W) and normal probability plots) and homogeneity of variances and log transformations were used when data violated one of the assumptions. For common terns, organ weights, bone lengths and biochemical endpoints measured on common tern hatchlings were analyzed using one-way analysis of variance (general linear model) followed by Tukey's HSD method of multiple comparison (α=0.05 for statistical significance, and $0.051<\alpha<0.075$ for marginal significance). For kestrels, since there were only two groups, endpoints were compared using one-way analysis of variance (general linear model which is analogous to the Student's t-test). As with the tern study, statistical significance was set at α=0.05, and marginal significance encompassed $0.051<\alpha<0.075$. A single suspect outlier in the kestrel study was evaluated using both Grubb's test and Cooks D statistic. Cumulative percent hatch was calculated as the number of chicks that hatched to up a given point in time divided by the final number of chicks hatching within each treatment group.

Results

Composition of DE-71 mixtures

As expected, the composition of neat DE-71 from Great Lakes Chemical Corporation and LGC Promochem mixture contained three dominant congeners (BDE-47, -99 and -100) which accounted for 88.28% and 89.71% of these mixtures respectively (Table 1). The quantities of the other 47 detected congeners were generally quite comparable between the two mixtures, and similar to detailed analyses of this commercial formulation (La Guardia et al. 2006). The

Table 1. Congener composition of neat DE-71 and dosing solutions[a]

Congener	Neat DE-71 from Great Lakes Chemical Corporation	Neat DE-71 from LGC Promochem	Dosing Solution Great Lakes Chemical Corporation (Present Study)	Dosing Solution from Promochem (McKernan et al. 2009, 2010)
BDE 30 (2,4,6 tri-BDE)	0	0	0	-
BDE 32 (2,4',6 tri-BDE)	0	0	0	-
BDE 17/25 (2,2',4 & 2,3',4 tri-BDE)	0.03	0.04	0	0.07[b]
BDE 28/33 (2,4,4' & 2',3,4 tri-BDE)	0.14	0.18	0.20	0.36
BDE 35 (3,3',4 tri-BDE)	0	0	0	ND
BDE 37 (3,4,4' tri-BDE)	0	0	0	ND
BDE 75 (2,4,4',6 tetra-BDE)	0.02	0.02	0	0
BDE 51 (2,2',4,6' tetra-BDE)	0.07	0.04	0	0.01
BDE 49 (2,2',4,5' tetra-BDE)	0.29	0.34	0.31	0.34
BDE 71/48 (2,3',4,6' & 2,2'4,5 tetra-BDE)	0.03	0.03	0	0.01
BDE 47 (2,2',4,4' tetra-BDE)	29.54	30.82	28.29	21.98
BDE 74 (2,4,4',6 tetra-BDE)	0.06	0.07	0	0.13
BDE 66/42 (2,3',4,4' & 2,2'3,4' tetraBDE)	0.21	0.22	0.257	0.70
BDE 104/121 (2,2',4,6,6' & 2,3',4,5',6 penta-BDE)	0.02	0.03	0	ND
BDE 102 (2,2',4,5,6' penta-BDE)	0.06	0.02	0	0.05
BDE 100 (2,2',4,4',6 penta-BDE)	8.56	10.65	9.52	17.53
BDE 101/120 (2,2',3,5,5' & 2,3',4,5,5'penta-BDE)	0.08	0.07	0	0.34[c]
BDE 119 (2,3',4,4',6 penta-BDE)	0.02	0.02	0	0.06
BDE 88 (2,2',3,4,6 penta-BDE)	0.06	0.02	0	ND
BDE 99 (2,2',4,4',5 penta-BDE)	50.18	48.24	51.95	37.29
BDE 116 (2,3,4,5,6 penta-BDE)	0	0	0	-
BDE 97/118 (2,2',3',4,5 & 2,3',4,4',5 penta-BDE)	0.02	0.03	0	ND
BDE 85 (2,2',3,4,4'penta-BDE)	1.22	1.64	1.55	4.39

BDE 126 (3,3',4,4',5 penta-BDE)	0	0	0	-
BDE 155 (2,2',4,4',6,6' hexa-BDE)	0.10	0.09	0	0.26
hexa BDE (b)	0	0	0	-
BDE 154 (2,2',4,4',5,6'hexa-BDE)	3.40	3.28	2.41	6.67
BDE 144 (2,2',3,4,5',6 hexa-BDE)	0	0	0	-
hexa bde (a)	0.04	0	0	-
BDE 153 (2,2',4,4',5,5'hexa-BDE)	4.30	3.15	3	6.35
BDE 139 (2,2',3,4,4',6 hexa-BDE)	0.71	0.50	1	1.48
BDE 140 (2,2',3,4,4',6' hexa-BDE)	0.16	0.09	0	0.34
hexa BDE (c)	0.04	0.02	0	-
BDE 138 (2,2',3,4,4',5',6 hexa-BDE)	0.50	0.27	1	0.90
BDE 156 (2,3,3',4,4',5 hexa-BDE)	0	0	0	-
BDE 188 (2,2',3,4',5,6,6' hepta-BDE)	0	0	0	-
BDE 184 (2,2',3,4,4',6,6' hepta-BDE)	0	0	0	0.06
BDE 179 (2,2',3,3',5,6,6' hepta-BDE)	0	0	0	-
BDE 128 (2,2',3,3',4,4' hexa-BDE)	0	0	0	-
BDE 175 (2,2',3,3',4,5',6 hepta-BDE)	0	0	0	-
BDE 183 (2,2',3,4,4',5',6 hepta-BDE)	0.13	0.14	0	0.60
BDE 182 (2,2',3,4,4',5,6' hepta-BDE)	0	0	0	-
BDE 185 (2,2',3,4,5,5',6 hepta-BDE)	0	0	0	-
BDE 192 (2,3,3',4,5,5',6 hepta-BDE)	0	0	0	-
BDE 191 (2,3,3',4,4',5',6 hepta-BDE)	0	0	0	-
BDE 180 (2,2',3,4,4',5,5' hepta-BDE)	0	0	0	-
BDE 181 (2,2',3,4,4',5,6 hepta-BDE)	0	0	0	-
BDE173/190(2,2',3,3',4,5,6 & 2,3,3',4,4',5,6 hepta-BDE)	0	0	0	-
BDE 171 (2,2',3,3',4,4',6 hepta-BDE)	0	0	0	-
penta (a)	-	-	-	0.07

[a] All values are expressed in percentages relative to the total PBDE detected in the mixture. ND=congener not detected; - = congener was not quantified.

[b] Congeners did not co-elute. BDE 17=0.07%; BDE-25 not analyzed for.

[c] Congeners did not co-elute. BDE 101=0.34%; BDE 120 = ND

congener composition of the DE-71 dosing solutions used in the present study was quite comparable to those used in previous work (McKernan et al. 2009, 2010) (BDE-47 28.29% vs. 21.98%; BDE-99 51.95% vs. 37.29%; and BDE-100 9.52% vs. 17.53%).

For the dosing solutions used in the present study, nominal concentrations were 0.2, 2 and 20 µg DE-71 per gram egg (injection volume of 0.5 µl corn oil vehicle per gram egg). Recoveries were 90%, 116% and 128%, respectively. Therefore, the actual amount of DE-71 in these solutions was 0.18, 2.32, 25.7 µg DE-71 per gram egg.

Background contamination of common tern and American kestrel eggs

All six control common tern eggs collected from Poplar Island, MD submitted for chemical analyses exhibited low concentrations of organochlorine pesticides (*p,p*'-DDE, DDMU, *cis*-nonachlor, *trans*-nonachlor, MC 5, mirex, and hexachlorobenzene; all <0.08 µg/g ww), total PCBs (<0.45 µg/g ww), and total PBDEs (<0.052 µg/g ww). There was poor recovery of analytes in one common tern egg sample, and thus it was not included in the calculation. Likewise, concentrations of organochlorine contaminants (<0.07 µg/g ww) from the three control eggs from the PWRC American kestrel colony were quite low. Concentrations of total PBDEs in kestrel eggs (<0.004 µg/g ww) were 10% or less of those found in common terns (Table 2 and 3).

Other organochlorine pesticides that were analyzed for and not detected in control common tern or American kestrel eggs were: *o,p'*-DDE, *o,p'* DDD, *p,p'* DDD, *p,p'* DDT, total BHC isomers (alpha, beta, gamma and lindane), total chlordanes (compound C, hepatachlor, MC1, MC2, MC3, MC6, MC7, MC8, *trans*-chlordane, heptachlor epoxide isomer B, oxychlordane, *cis*-chlordane, compound K), aldrin, dieldrin, metholxychlor, methoxychlor olefin, pentachloroanisole, methoxy triclosan, endosulfan (endosulfan I, II, endosulfan sulfate),

Table 2. Predominant organochlorine pesticides and PCBs in control eggs

	Common Tern Poplar Island (n=6[a])	American Kestrel PWRC Colony (n=3)
Contaminant (µg/g)	Mean Extremes n detected	Mean Extremes n detected
p,p' -DDE	0.06 0.029-0.076 5	0.002 0.0019-0.002 3
DDMU	- ND-0.003 1	- ND 0
cis -Nonachlor	0.002 ND-0.003 4	- ND 0
trans -Nonachlor	0.002 ND-0.006 4	- ND 0
MC 5	0.001 ND-0.004 3	- ND 0
Mirex	0.002 ND-0.005 4	- ND 0
Hexachlorobenzene	0.002 0.001-0.002 5	0.001 0.0010-0.0014 3
Total PCBs	0.31-0.44 6	0.034-0.07 3

[a] Out of the 6 control eggs analyzed, one had poor recovery for organochlorine pesticides and was not inculded in the calculations.

Table 3. Total polybrominated diphenyl ether and congeners

	Common Tern Poplar Island (n=6[a])	American Kestrel PWRC Colony (n=3)
Contaminant (ng/g)	Mean Extremes n detected	Mean Extremes n detected
Total PBDEs	22.8 10.2-51.0 5	2.83 1.88-3.47 3
BDE congener 47	10.6 5.57-20.3 5	0.61 0.54-0.67 3
BDE congener 49	- ND-0.25 1	- ND 0
BDE congener 85	- ND-0.84 1	- ND 0
BDE congener 99	6.96 2.02-20.6 5	0.64 0.41-0.87 3
BDE congener 100	3.46 2.16-5.73 5	0.26 0.19-0.33 3
BDE congener 153	- ND-1.86 2	1.13 0.75-1.33 3
BDE congener 154	1.03 ND-2.02 4	0.212 ND-0.30 2

[a] Out of the 6 control eggs analyzed, one had poor recovery for PBDEs and was not inculded in the calculations.

endrin and metabolites (endrin-ketone, endrin-aldehyde), octachlordibenzodioxin, and octachlordibenzofuran.

Survival to 90% Incubation, Pipping and Hatching Success

For vehicle injected common tern eggs (control group), survival to 90% of incubation, and pipping and hatching success, were 87.5%, 81.2% and 81.2%, respectively (Table 4). Moisture loss (n=40 hatched eggs) averaged 14.5 % by the time eggs reached the pipping stage. There were no significant differences for survival, pipping and hatching success endpoints among the DE-71 dose levels and controls (p>0.14). For American kestrel vehicle injected eggs (control group), survival to 90% of incubation, pipping and hatching success were 96.9%, 93.8%, 87.5% respectively. Moisture loss (n=50 hatched eggs) averaged 13% by the time eggs reached the pipping stage. Unlike the common terns, DE-71 treated kestrel eggs had greatest mortality between the pipping stage and hatch. However, there were no statistically significant differences in survival to 90% of incubation, pipping and hatching success between controls and DE-71 treated eggs (p>0.15).

Mortality occurred early in incubation in the common terns, but near the end of incubation in kestrels. For common tern eggs, most of the losses occurred between days 5 to 12 of incubation in the 2 and 20 µg DE-71 per g egg doses (4 eggs lost at each dose), but there were very few losses in any group thereafter (Table 4). There was no difference in survival from pip to hatch between controls (13/13) and DE-71 treated eggs (27/28) (p=1.00). Unlike common terns, there was a pronounced decrease in survival from pip to hatch in DE-71-treated kestrels. Of the 29 DE-71 treated kestrel eggs that pipped, 7 failed to hatch, while of the 30 control eggs

Table 4. Survival of embryos to 90% incubation, pipping and to hatch. Fractions represent number that made it to stage over total sample size.

Species	Endpoint	Dose							
		Controls		0.2 µg/g DE-71		2 µg/g DE-71		20 µg/g DE-71	
		Successful/n	%	Successful/n	%	Successful/n	%	Successful/n	%
Common Tern	Survival to 90%	14/16	87.5	10/11	90.9	7/11	63.6	12/16	75
	Pipped	13/16	81.2	9/11	81.8	7/11	63.6	12/16	75
	Hatched	13/16	81.2	8/11	72.7	7/11	63.6	12/16	75
American kestrel	Survival to 90%	31/32	96.9					29/30	96.67
	Pipped	30/32	93.8					29/30	96.67
	Hatch	28/32	87.5					22/30	73.33

that pipped, only 2 failed to hatch (p=0.08). Comparison of losses from pip to hatch stage was marginally different between the two species (p=0.052).

There was no dose-response relation between quantity of DE-71 administered and time to hatch in common terns (Figure 14a). However, when all DE-71 treatment groups were combined into a single group, the DE-71-treated tern eggs hatched 0.44 days later than the vehicle-injected controls (21.59 ± 0.115 vs. 22.03 ± 0.105 days , p=0.014) (Figure 15a). A different pattern was observed in American kestrels (Figure 14b). The first 8 eggs to hatch were all controls (Fisher's Exact Test, p=0.0064). However, by the end of the study, there was no difference in time to hatch between controls and DE-71-treated kestrel eggs (27.7 ± 0.123 vs. 27.9 ± 0.124, p=0.29) (Figures 14b and 15b).

Organ weights and bone lengths

No gross deformities were observed in common tern or kestrel hatchlings. However, three DE-71 treated kestrels that failed to hatch exhibited some edema in the head/neck region. For common terns, crown-rump lengths, and the weight of the whole embryo, yolk sac, liver, bursa, thyroids, and the body to organ weight ratios for liver, bursa, and thyroids did not differ significantly among the four groups (Table 5). For total thyroid weight, the overall p-value for ANOVA, was 0.0652; however, Tukey's HSD post-hoc test did not reveal any differences among groups at the marginally significant (p=0.075) level. Examination of the descriptive statistics suggested that spleen weight was greater in the DE-71 treated hatchlings than controls. The analysis of variance and the multiple comparison procedure revealed that both spleen weight and spleen to body weight ratio were greater in the 2 µg/g DE-71 dose compared to the controls (p=0.05 and 0.03, respectively), but no such difference was detected for other comparisons. Crown-rump length, body, yolk sac, liver, spleen, and bursa weights and organ to body

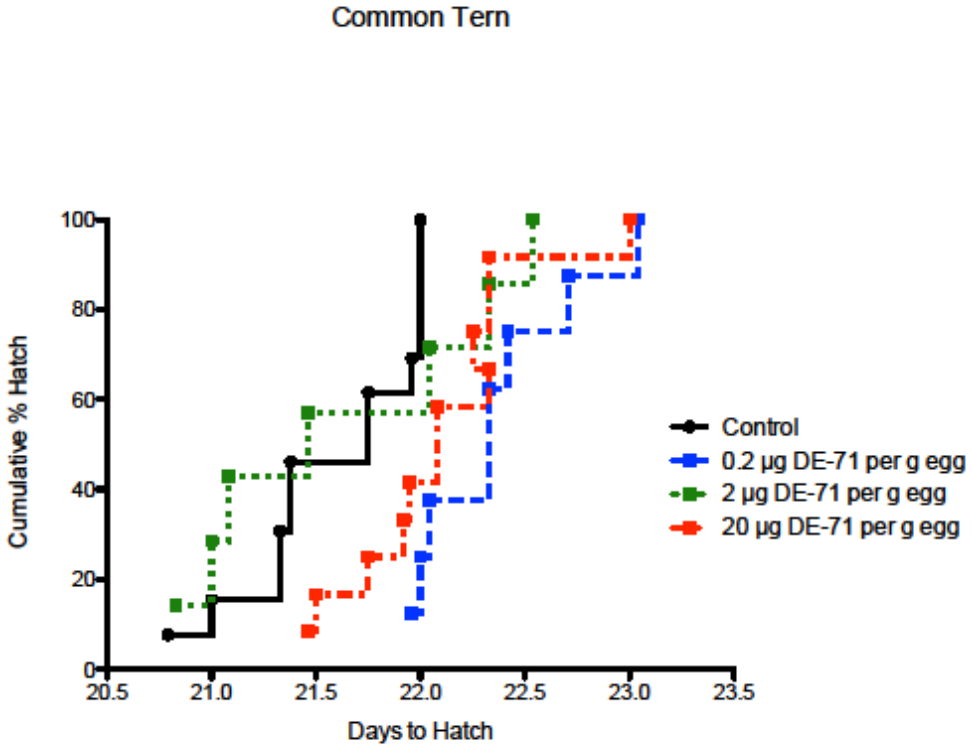


Figure 14a. Cumulative percent hatch for common terns

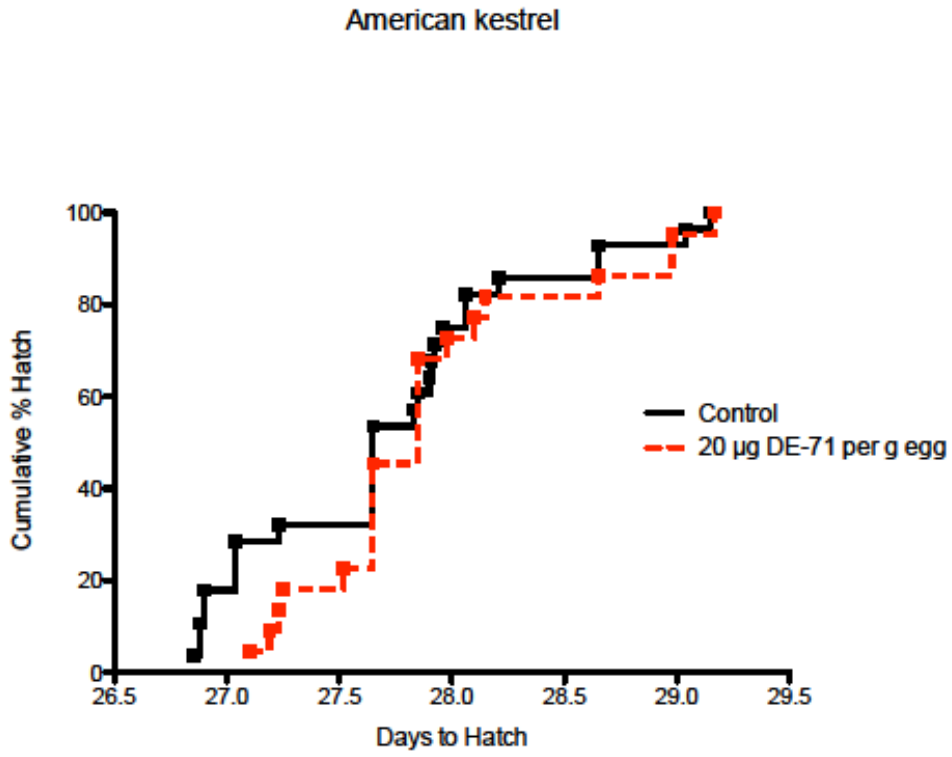


Figure 14b. Cumulative percent hatch for American kestrels

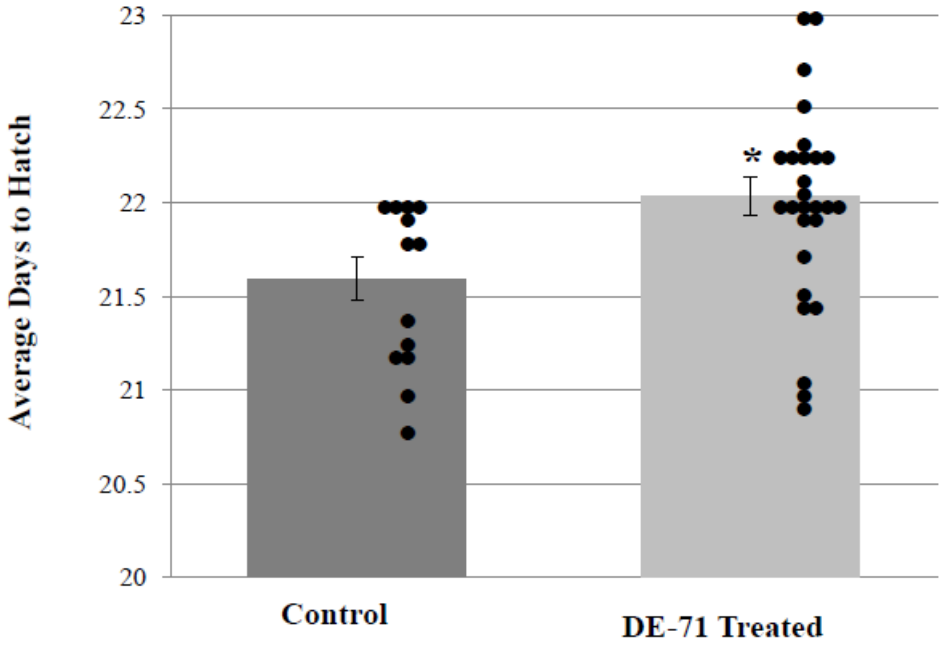


Figure 15a. Average days to hatch for common terns. *=p<0.05

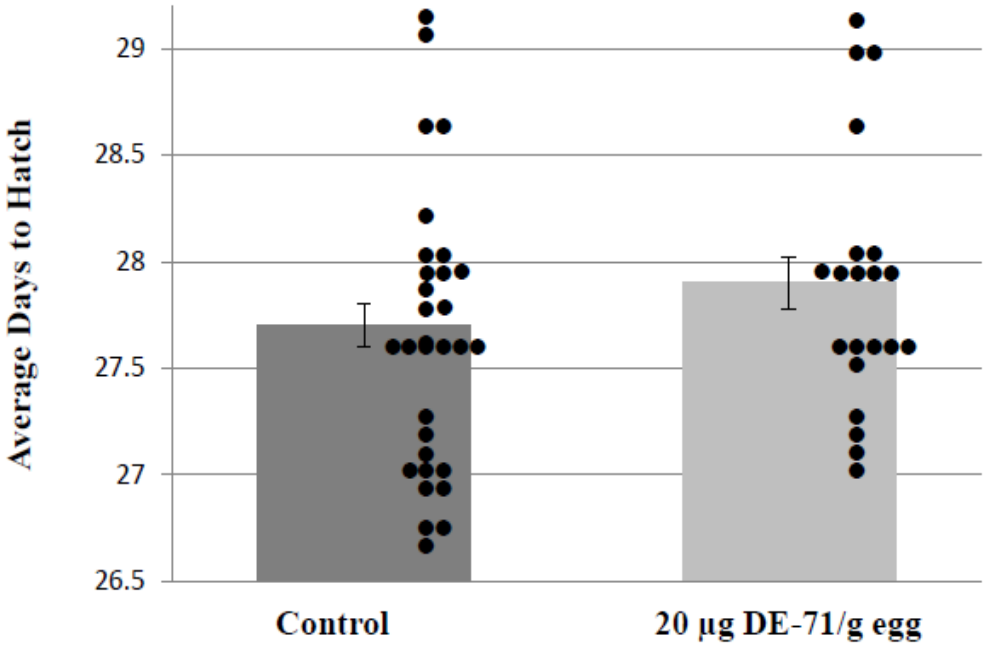


Figure 15b. Average days to hatch for American kestrels.

Table 5. Morphological endpoints and bone lengths for terns and kestrels[1]

Species	Morphological Endpoints	Dose			
		Controls	**0.2 µg DE-71/g egg**	**2 µg DE-71/g egg**	**20 µg DE-71/g egg**
		n=13	**n=8**	**n=7**	**n=12**
Common Tern	Crown-Rump Length (cm)	7.96 ± 0.119	7.71 ± 0.067	7.99 ± 0.040	7.83 ± 0.061
	Body Weight w/ Yolk Sac (g)	13.87 ± 0.359	13.01 ± 0.343	13.77 ± 0.210	14.11 ± 0.310
	Yolk Sac (g)	1.32 ± 0.143	1.25 ± 0.171	1.25 ± 0.122	1.64 ± 0.228
	Body Weight w/o Yolk Sac (g)	12.55 ± 0.264	11.76 ± 0.229	12.52 ± 0.236	12.47 ± 0.204
	Liver (mg)	402.0 ± 12.06	375.8 ± 24.15	360.6 ± 26.77	391.1 ± 9.56
	Spleen (mg)	7.51 ± 0.709[B,2]	8.10 ± 0.024[AB]	10.96 ± 1.417[A]	9.95 ± 0.778[AB,4]
	Bursa (mg)	14.51 ± 0.921	14.90 ± 0.702	13.93 ± 1.133	13.68 ± 0.696
	Total Thyroid (mg)	3.14 ± 0.274	2.23 ± 0.094[3]	3.20 ± 0.190	2.75 ± 0.238
	Left Thyroid (mg)	1.45 ± 0.124	0.99 ± 0.15[3]	1.60 ± 0.133	1.38 ± 0.170
	Right Thyroid (mg)	1.68 ± 0.204	1.175 ± 0.13	1.60 ± 0.229	1.38 ± 0.109
	Liver: Body Weight x 100	3.20 ± 0.665	3.18 ± 0.164	2.89 ± 0.235	3.91 ± 0.676
	Spleen: Body Weight x 100	0.06 ± 0.005[B,2]	0.07 ± 0.006[AB]	0.09 ± 0.012[A]	0.08 ± 0.006[B,4]
	Bursa: Body Weight x 100	0.12 ± 0.007	0.13 ± 0.010	0.11 ± 0.011	0.11 ± 0.006
	Total Thyroid: Body Weight x 100	0.025 ± 0.0019	0.019 ± 0.0007[3]	0.026 ± 0.0016	0.022 ± 0.0019
	Right Thyroid: Body Weight x 100	0.013 ± 0.0015	0.010 ± 0.0012	0.013 ± 0.0018	0.011 ± 0.0008
	Left Thyroid: Body Weight x 100	0.012 ± 0.0009	0.008 ± 0.0012[3]	0.013 ± 0.0012	0.011 ± 0.0014
	Bone Lengths (mm)				
	Humerus	8.88 ± 0.117	8.62 ± 0.134	8.49 ± 0.219	8.79 ± 0.078
	Ulna	9.03 ± 0.139	8.69 ± 0.134	9.12 ± 0.162	8.96 ± 0.127
	Femur	9.86 ± 0.089	9.55 ± 0.187	9.94 ± 0.246	9.73 ± 0.102
	Tibiatarsus	16.19 ± 0.184	15.89 ± 0.259	16.50 ± 0.271	16.19 ± 0.214
	Metatarsus	8.99 ± 0.109	8.64 ± 0.186	8.94 ± 0.300	8.57 ± 0.097

		Controls	**20 µg DE-71/g egg**
		n=28	**n=22**
American kestrel	Crown-Rump Length (cm)	6.06 ± 0.042	6.06 ± 0.027
	Body Weight w/ Yolk Sac (g)	10.68 ± 0.191	10.33 ± 0.156
	Yolk Sac (g)	0.87 ± 0.060[5]	0.78 ± 0.061
	Body Weight w/o Yolk Sac (g)	9.84 ± 0.146	9.55 ± 0.134
	Liver (mg)	256.9 ± 8.921	267.4 ± 7.945
	Spleen (mg)	7.63 ± 0.457	6.93 ± 0.387[7]
	Bursa (mg)	11.56 ± 0.541	12.31 ± 0.654
	Total Thyroid (mg)	3.096 ± 0.177[6]	2.74 ± 0.139[*]
	Right Thyroid (mg)	1.66 ± 0.117[5]	1.49 ± 0.118
	Left Thyroid (mg)	1.49 ± 0.083[6]	1.24 ± 0.095[†]
	Liver: Body Weight x 100	2.59 ± 0.074	2.79 ± 0.072[†]
	Spleen: Body Weight x 100	0.08 ± 0.004	0.07 ± 0.004[7]
	Bursa: Body Weight x 100	0.12 ± 0.006	0.13 ± 0.007
	Total Thyroid: Body Weight x 100	0.032 ± 0.0013[5]	0.027 ± 0.0014[†]
	Right Thyroid: Body Weight x 100	0.017 ± 0.0011[4]	0.016 ± 0.0009
	Left Thyroid: Body Weight x 100	0.015 ± 0.0008[5]	0.013 ± 0.0010
	Bone Lengths (mm)		
	Humerus	6.96 ± 0.045[*,8]	6.67 ± 0.087
	Ulna	6.69 ± 0.08	6.71 ± 0.068
	Femur	8.59 ± 0.085	8.63 ± 0.106
	Tibiatarsus	9.75 ± 0.101	9.79 ± 0.130
	Metatarsus	6.91 ± 0.084	6.82 ± 0.101

[1]Organ to body weight (w/o yolk sac) ratios x 100. For terns, groups with the same letter superscripts are not significantly different $p>0.05$.
For kestrels, *=$p<0.05$; †=$p<0.075$.
[2]n=12, [3] n=7, [4]n=11, [5] n=27, [6] n=26, [7] n=21, and [8] humerus length after removal of outlier.

weight ratios did not differ between DE-71-treated and control kestrel hatchlings. Total thyroid weight was significantly less in DE-71-treated kestrels compared to controls (p=0.0355). However, the thyroid to body weight ratio was only marginally lower in treated birds (p<0.075).

In common tern hatchlings, the length of the tibiotarsus, metatarsus, femur, humerus and ulna did not differ among groups (p>0.17). For kestrels, tibiotarsus, metatarsus, femur and ulna length did not differ between controls and the 20 µg DE-71/g - treated eggs (p>0.49). The variances for humerus length were homogeneous, but the data deviated from normality (Shapiro Wilk test, p=0.0046). However, humerus length was shorter in hatchings from the DE-71 treated eggs than vehicle injected control kestrels (p=0.029). When the single outlier in the control group was censored, data became normally distributed (Shapiro Wilk test, p = 0.774), and this difference in humerus length remained significant (p=0.002).

Histopathology

A preliminary evaluation of hematoxylin and eosin stained sections did not reveal any dramatic changes in liver, kidney, spleen, bursa, and thyroids of hatchling common terns or American kestrels that had been treated with DE-71. In a more detailed evaluation of a subset of the common tern samples, liver and thyroids were unremarkable and generally uniform among control and DE-71 treated hatchlings, with the exception of minimal thyroid follicular hyperplasia in one hatchling in the 2 µg/g group. A seemingly increased prevalence of splenic hematopoiesis was apparent in DE-71 hatchlings (6 of 12 samples in the 20 µg DE-71 per g egg doses) compared to controls (1 of 5 samples), but this was not statistically significant (p>0.252). For other tissues, changes were modest and not unusual for hatchling birds, and included the

occasional presence of heterophilic granulocytes in kidney, and lymphocyte proliferation in spleen and bursa.

Oxidative Stress

There were no statistically significant differences in oxidative stress endpoints in liver tissue of common tern hatchlings ($p > 0.650$) (Table 6). For kestrel hatchlings, means of all oxidative stress endpoints were numerically greater in the DE-71 treatment group compared to the vehicle injected control group. Difference were significant ($p<0.05$) for the concentration of oxidized glutathione and marginally significant ($p<0.07$) for reduced glutathione and TBARS.

Discussion

Background contamination of common tern and American kestrel eggs

In the six common tern eggs collected from Poplar Island, MD in 2010, and used as controls in our study, background concentrations of organochlorine pesticides (< 0.08 µg/g ww), PCBs (<0.45 µg/g ww) and PBDEs (<0.052 µg/g ww) were very low and well-below known reproductive effect thresholds in birds. For common terns nesting in Chesapeake Bay, there are limited contaminant exposure data. Most of the ecotoxicological data for common terns nesting in Chesapeake Bay have been collected from tern colonies in remote locations. As part of an egg injection study (Hoffman et al. 1998), ten common tern eggs were collected from South Sand Point off of Barren Island in 1994 and analyzed for organochlorine contaminants. Eggs contained moderate levels of *p,p'*-DDE (0.04-0.22 µg/g ww) and *trans*-chlordane (not detected up to 0.06 µg/g ww). All mean levels of PCBs were less than 2.47 µg/g ww (J.B. French, USGS

Table 6. Oxidative stress endpointsfor terns and kestrels

Species	Endpoints	Dose			
		Controls	**0.2 µg DE-71/g egg**	**2 µg DE-71/g egg**	**20 µg DE-71/g egg**
		n=13	**n=8**	**n=7**	**n-12**
Common Tern	Total Sulfhydryl (TSH) (µmol/g)	42.6 ± 2.54	41.7 ± 3.11	46.1 ± 3.23	43.9 ± 1.58
	Protein Bound Sulfhydryl (PBSH) (µmol/g)	34.56 ± 2.25	33.9 ± 2.76	37.96 ± 4.10	35.91 ± 1.41
	Total Glutathione (TotGSH) (µmol/g)	10.04 ± 0.47	9.74 ± 0.57	10.23 ± 0.58	9.97 ± 0.28
	Reduced Glutathione (GSH) (µmol/g)	8.06 ± 0.35	7.78 ± 0.37	8.16 ± 0.41	7.95 ± 0.20
	Oxidized Glutathione (GSSG) (µmol/g)	0.99 ± 0.12	0.98 ± 0.14	1.04 ± 0.13	1.01 ± 0.07
	Ratio of GSSG to GSH	0.12 ± 0.01	0.13 ± 0.02	0.13 ± 0.01	0.13 ± 0.01
	TBARS (nmol/g)	4.94 ± 0.73	4.33 ± 0.59	4.10 ± 0.37	4.30 ± 0.29
		n=28			**n=22**
American kestrel	Total Sulfhydryl (TSH) (µmol/g)	25.8 ± 1.05			30.0 ± 1.82
	Protein Bound Sulfhydryl (PBSH) (µmol/g)	20.63 ± 0.94			24.31 ± 1.60
	Total Glutathione (TotGSH) (µmol/g)	6.99 ± 0.23			7.92 ± 0.39
	Reduced Glutathione (GSH) (µmol/g)	5.15 ± 0.13			5.65 ± 0.24†
	Oxidized Glutathione (GSSG) (µmol/g)	0.92 ± 0.06			1.13 ± 0.08*
	Ratio of GSSG to GSH	0.18 ± 0.01			0.20 ± 0.01
	TBARS (nmol/g)	2.48 ± 0.13			2.89 ± 0.20†

*=p <0.05; † = p < 0.07

Patuxent Wildlife Research Center, unpublished data, accessible in CEE-TV database, Rattner et al. 2005). In 1997, thirty common tern eggs were collected from Bodkin Island. All concentrations of organochlorine pesticides were below the detection limit (0.061 µg/g dw) and PCBs ranged from 0.44-1.50 µg/g ww (French et al. 2001, Rattner et al. 2007).

Data on PBDE residues in eggs of raptors (ospreys, peregrine falcons) nesting in Chesapeake Bay indicate their presence in much greater concentrations particularly in urbanized and industrialized locations (Rattner et al. 2004, Potter et al. 2009). For example, Rattner et al. (2004) reported levels of PBDEs that approached both the lowest adverse effect levels for survival in kestrels (1.8 µg DE-71 per g egg ww) and that observed in Henny et al. (2009, 2011) in which osprey productivity was suggested to be inversely related at PBDE concentrations >1 µg/g ww. As part of a current Chesapeake Bay ecotoxicological investigation, samples are being collected to examine temporal trends and the relationship between concentrations of organochlorine pesticides, PCBs, PBDEs, and other contaminants with reproductive success in ospreys. In view of the findings of the present tern study, Poplar Island is being used as a reference site.

In 2000-2003, Forster's tern eggs collected from San Francisco Bay were found to have concentrations of PBDEs that ranged up to 63 µg/g lipid weight on a dry basis (She et al. 2008). Using these data (She et al. 2008), we estimated wet weight concentrations by adjusting for lipid and moisture loss during incubation followed by a conversion of lipid content on a dry weight basis back to that of a fresh egg. Annual average PBDE concentration estimates in Forster's tern eggs ranged from 0.39 to 1.56 µg DE-71 per g ww. Polybrominated diphenyl ether residues in Forster's tern eggs from San Francisco Bay were nearly 2 orders of magnitude greater than the residues in common tern eggs we collected from Poplar Island. These data indicate that the tern

eggs collected from Poplar Island were suitable for evaluating PBDE effects (i.e., other measured compounds were unlikely to confound the effects of the DE-71). Poplar Island may be an appropriate source of uncontaminated bird eggs for use in monitoring and other toxicity studies.

Not unexpectedly, kestrel eggs from the captive colony at the Patuxent Wildlife Research Center had very low concentrations of organochlorine pesticides and metabolites, total PCBs, and PBDEs; the range of concentrations was similar to that reported in other studies using eggs from this colony (e.g., Wiemeyer et al. 2001, McKernan et al. 2010).

Use of Egg Injection Studies to Evaluate Toxicity of DE-71

For well over a century, the avian egg has been used in toxicity testing (chemical screening, comparative species sensitivity, and mechanistic studies). Although the introduction of a contaminant into an egg by way of egg injection does not perfectly mimic natural exposure and deposition of contaminants into the contents of an egg (yolk and albumen), it does serve as a moderately inexpensive technique to evaluate embryotoxicity and teratogenicity of a variety of compounds. Recent egg injection studies from our own lab have investigated PCBs, methylmercury, and PBDEs (e.g., Hoffman et al. 1998, Heinz et al. 2009b, McKernan et al. 2007, 2009, 2010).

Unlike some compounds such as methylmercury, where the majority of the compound makes its way from the air cell into the egg, only a fraction of the air cell administered PBDE dose makes its way into the contents of the egg by the time of pipping (chicken 29.6%, mallard 27.7%, kestrel 18.8%, black-crowned night-heron (*Nycticorax nytcicorax*) (21.8 %) (McKernan et al. 2010). In the present study, we did not rigorously determine the uptake of DE-71 into the contents of common tern eggs. However, based on the amount of the dose that was actually

transferred across the air cell membrane into the yolk, albumen and embryo of the avian species studied by McKernan et al. (2010), it is likely that approximately 18.8-29.6% of the air cell administered dose was transferred into tern egg contents over the course of incubation. Thus, at the greatest dose (analytically determined to be 25.7 µg DE-71 per g egg), approximately 4.8 to 7.6 µg DE-71 per g of egg may have actually entered the egg contents. This range of concentrations exceeds the average values of PBDEs found in Caspian terns (*Sterna caspia*), Forster's terns, least terns (*Sterna antillarum brownii*), and California clapper rails (*Rallus longirostris obsoletus*) from San Francisco Bay by a factor of 4.4 to 6.9 (She et al. 2008). In DE-71 egg injection studies, the transfer of the compound into the egg contents occurs gradually over the course of incubation (congeners with the fewest bromine atoms and lowest $LogK_{ow}$ entering more rapidly; McKernan et al. 2010), while in nature, the chemical is maternally deposited directly into the yolk and present throughout the entire incubation period. Such factors could account for differences in sensitivity of compounds tested in egg injection studies versus eggs from free-ranging birds.

In an attempt to optimize the moisture loss that occurred during incubation of our tern eggs, the rate of moisture loss was carefully monitored and adjusted in an attempt to steer each egg toward a loss of approximately 16% of its pre-incubated fresh weight by the time of pipping. If an egg does not loose enough moisture, the embryo will be wet and emerge from the shell with greater difficulty. Extra efforts were taken to adjust the rate of moisture loss by means of transferring eggs among separate incubators, each set at a different relative humidity. In the present study, moisture lost by pipping in common terns and kestrels (14.5 and 13.0% respectively) approached the 16% target. A subset of four control kestrel eggs incubated by McKernan and coworkers (2009) exhibited an average of 15.5% moisture loss. Thus, it seems

unlikely that the reduced hatching success observed by McKernan and coworkers (2009) at 10 and 20 µg DE-71 per g egg dosages was to improper incubation conditions.

Survival to 90% Incubation, Pipping and Hatching Success

In the present study, artificially incubated control common tern and kestrel eggs exhibited good survival through 90% of incubation (87.5 and 96.9%, respectively), pipping success (81.2 and 93.8%), and hatching success (81.2 and 87.5%). These rates are not unlike hatching success observed in other studies using kestrel and tern eggs conducted at our laboratory (e.g., Heinz et al. 2009, Hoffman et al. 1998, McKernan et al. 2009).

Administration of 0.2, 2, and 20 µg DE-71 per gram of egg into the air cell of common tern eggs did not adversely affect survival to 90% incubation, pipping, and hatching success. Based on survival endpoints, common terns are apparently more tolerant to air cell administered DE-71 than kestrels, but similar in sensitivity as to chickens, mallards, and black-crowned night-herons (McKernan et al. 2009, 2010). Mortality of developing tern embryos principally occurred during the first half of incubation rather than at pip and hatch, which differs from the time-course of loss of DE-71-treated kestrel eggs in the present study and also in the study by McKernan et al. 2009. It is possible that this difference in the timing of mortality could reflect more rapid transfer of DE-71 across the air cell membrane in tern embryos compared to kestrels; for many toxicants, early developmental stages in bird eggs are the most sensitive period for embryotoxicity.

The kestrel egg injection study was undertaken in order to replicate findings of McKernan et al. (2009), serving in some sense as a positive control for our common tern study. In contrast to previous findings (McKernan et al. 2009), administration of 20 µg DE-71 per gram egg to kestrels did not affect pipping or hatching success, but nonetheless, subtle effects (the first

8 eggs to hatch were all controls) were detected that suggest DE-71 delayed egg hatching. Pipping and hatching of kestrel eggs was then monitored very closely to estimate days to hatch, as delayed hatching due to DE-71 might reflect delayed development or a weakened state of the embryo. This could place DE-71 exposed altricial hatchlings at a survival disadvantage. However, by the end of incubation for kestrels, the DE-71 treated eggs did not differ in hatch time compared to controls. The present findings in DE-71 kestrels differ from the dose-dependent pipping and hatching failure observed by McKernan and coworkers (2009), but certainly do not invalidate the earlier results.

Unlike altricial kestrels, terns nest in open areas and their nests consist of no more than a small scrape. Upon hatching, semi-precocial tern chicks must be able to consume small food items within 1-3 hours of hatching and seek shelter in nearby vegetation by 2-3 days post-hatch in order to avoid predation and overheating. The delayed hatching of DE-71-treated tern eggs may have implications for post-hatching survival of precocial wild birds. A delay in hatch could result in weakened or exhausted tern chicks, that might result in a fitness and survival disadvantage (e.g., less able to seek vegetative cover, and be more vulnerable to predation or other environmental factors).

Organ Weights, Bone Lengths, Deformities and Histopathological Examinations

In agreement with our previous studies (McKernan et al. 2009, 2010), DE-71 did not evoke teratogenic effects in our study. However, three DE-71 treated kestrels (but not common terns) that failed to hatch exhibited edema, a finding also observed in our earlier study (McKernan et al. 2009). For both common terns and American kestrel hatchlings, the majority of the organ weight and bone length measurement endpoints were not affected by the flame

retardant. Although not dose-dependent, there was some evidence of DE-71 enlargement of spleen, as reflected by absolute weight and spleen to body weight ratio for common terns in the 2 µg DE-71 per g egg group. Additional evidence of DE-71 effects on spleen was the qualitative suggestion of enhanced splenic hematopoisis in the 20 µg DE-71 per g egg group, but this was not borne out by statistical analysis. Taken together, changes in spleen were subtle, and probably transient and of limited biological consequence.

Kestrels that were treated with DE-71 had both shorter humerus lengths, and total thyroid weight was reduced. It is possible that smaller humerus length may have implications for hatching time (initially delayed in treated kestrels) and post-hatching survival. In rodent laboratory studies, PBDE exposure decreased thyroxine (T4) concentration (Fowles et al. 1994, Hallgren et al. 2001, Zhou et al. 2001), and slightly lower T4 concentrations have also been observed in PBDE-treated kestrel nestlings (Fernie et al. 2005b). Mechanistic studies suggest that PBDEs may alter plasma transport of T4 through competitive binding mechanisms (structure of thyronine hormones and hormone precursors are quite similar to the structure of some PBDE congeners) (Hallgren and Darnerud 2002) and the induction of T4 degradation activity (hepatic T4 glucouronidation activity) (Zhou et al. 2001; Richardson et al. 2008). The smaller thyroid weight observed in hatchlings could be a crude manifestation of such effects, although in our previous study (McKernan et al. 2009) no such effects were observed. Thyroid histology, often considered the "gold standard" of thyrotoxic effects, was unremarkable in both kestrels and common terns in the present study, a finding similar to that in other avian studies (Fernie et al. 2005b, McKernan et al. 2009).

Oxidative Stress

Oxidative stress is one mechanism by which brominated flame retardants evoke toxicity. The measurement of glutathione and antioxidant status, and lipid peroxidation, have been widely used as a biomarkers to study the exposure and potential adverse effects that brominated flame retardants can have on biological macromolecules, including lipids and DNA. Analysis of glutathione status, sulfhydryl concentrations and lipid peroxidation revealed differences in the responses to DE-71 between common terns and kestrels.

Common tern hatchlings did not exhibit any significant changes in oxidative stress endpoints, suggesting lower sensitivity compared to kestrels to the DE-71 formulation. In contrast to terns, a significant increase in the hepatic concentration of oxidized glutathione (GSSG) was observed in kestrels exposed to DE-71. Oxidation of glutathione (GSH) generally results in the accumulation of GSSG (Lu 1999) and exposure to brominated flame retardants may exacerbate this effect. Increased GSSG was also observed by Fernie et al. (2005b) in kestrels exposed to the principal congeners that make up the penta-BDE formulation.

However, in contrast to the findings in other BDE toxicity studies with kestrels (Fernie et al. 2005b) and earthworms (*Eisenia fetida;* Xie et al. 2011) which evoked responses that generally occur with severe oxidative stress, a marginal increase in glutathione (GSH) was observed in our DE-71 exposed kestrel hatchlings, thus leaving the GSSG to GSH ratio unchanged. The increase in GSH levels is potentially a result of *de novo* synthesis of GSH as a defense mechanism against reactive oxyradicals associated with DE-71 exposure. Our findings also suggest that compensatory mechanisms are activated, which stabilize the ratio between GSSG and GSH, as described in other studies (Xie et al. 2011).

In our study, common terns exhibited greater baseline levels of GSH than kestrels. Interestingly, a study in mice (*Mus musculus*) found that that DE-71 toxicity is mediated by oxidative stress and that toxicity is modulated by increased cellular GSH levels (Giordano et al. 2008). Knockout mice (those with a reduced ability to synthesize GSH) exposed to DE-71 had significantly lower concentrations of GSH than their wild-type counterparts, and were more susceptible to oxidative attack on biological macromolecules resulting cytotoxicity and apoptotic cell death (Giordano et al. 2008). These findings may imply that the greater GSH concentrations observed in terns are enough to counter DE-71 toxicity at our test doses, preventing any increase in oxidative stress above baseline values.

Hepatic thiols protect against the effects of oxidative stress, but are depleted under severe stress due to oxidation and binding with reactive species (e.g., Hoffman et al. 2005). For common terns no changes were observed in concentrations of total sulfhydryls (TSH) and protein bound sulfhydryls (PBSH). Although not statistically significant, both TSH and PBSH concentrations were consistently greater in the DE-71 treated kestrels compared to controls. In a previous study, Fernie et al. (2005b) reported a significant positive association of TSH and PBSH with BDE-183 and PBSH with BDE-99 in post-fledgling American kestrels. Thus, the thiol data are consistent with the TBARS and glutathione data indicating that the exposure conditions induced mild oxidative stress in kestrels.

Lipid peroxidation is a manifestation of oxidative damage and triggers the production of reactive intermediates that can cause protein and DNA damage (Monaghan et al. 2009), and contribute to the loss of cell function (Marnett 1999, Storey 1996). In our study, no changes in TBARS concentrations were observed in terns, consistent with the absence of changes in

glutathione status. Kestrels exhibited a marginal increase in TBARS suggesting that the level of oxidative stress, while elevated above that in controls, was slight.

Conclusion

Although the toxic effects of DE-71 on the development and hatching of kestrels were not as remarkable as were found in our previous work (McKernan et al. 2009), qualitative evaluation of a combination of measurement endpoints (weight of evidence approach) suggests that kestrels are more sensitive than common terns and several other species of birds (Table 7). Results from this study, conducted in the laboratory using controlled exposures, suggest that common tern embryos, and perhaps other tern species, are no more sensitive (and are probably less sensitive) to PBDEs than are kestrels. This pattern of embryotoxicity, with kestrels being more sensitive than common terns, has been observed in egg injection studies involving both methylmercury (Heinz et al. 2009b) and PCB congener 126 (Hoffman et al. 1998). An evaluation of the sensitivity of 25 terrestrial vertebrate species to various classes of contaminants (persistent organic pollutants, mercury, petroleum crude oil, and organophosphorous insecticides) using a combination of both laboratory and field data indicates that common terns are moderately sensitive (but not ultra sensitive) compared to other avian species (Golden and Rattner 2003).

The egg injection method evaluates intrinsic embryotoxic effects. Following air cell administration, DE-71 is transferred into egg contents gradually (McKernan et al. 2010), which is not unlike what occurs in nature (via assimilation of yolk during embryonic development) for many organic contaminants. However, in laboratory studies, the compound is not administered until after early morphogenesis, whereas in nature the toxicant is present in yolk from the onset of incubation. This test system does not evaluate extrinsic factors that can adversely affect

Table 7. Overview of effects on American kestrel and common tern embryos and hatchings exposed to DE-71[1]

	Chicken[2]	**Mallard**[2]	**Kestrel**[2]	**Kestrel** (Current Study)	**Common tern** (Current Study)
Survival to 90%	-	-	-	-	-
Delay in Hatch	NA	NA	NA	+	++
Pipping Success	-	-	++	-	-
Hatching Success	-	-	++	-	-
Edema in embryo or hatchling	-	-	-	+	-
Deformities	-	-	-	-	-
Bone Lengths	-	-	-	++	-
Histopathology	++	-	-	-	-
Spleen Weight and Spleen : Body Weight	-	-	NA	-	++
Thyroid Weight and Thyroid : Body Weight	-	-	NA	++	-
Thyroid Hormone	-	-	-	NA	NA
EROD	++	-	-	NA	NA
Oxidative Stress	NA	NA	NA	++	-

[1] - = no effect; NA=not analyzed; + = suspected effect, or marginally significant ; ++ = statistically significant effect (p <0.05)

[2] Results from McKernan et al. 2009

reproduction (e.g., courtship behavior, clutch size, nest attentiveness, and brood rearing) that have been observed with PCBs and PBDEs (e.g., Fox et al. 1978, Marteinson et al. 2010). Fortunately, the effects of DE-71 observed in both laboratory studies and in nature, are less dramatic at the molecular, organismal and population levels (Chen and Hale 2010, Henny et al. 2011) compared to other compounds, including methylmercury and PCBs.

Acknowledgements

The authors wish to thank R. Michael Erwin, Peter C. McGowan, Sarah E. Warner, Wayne C. Bauer and Mary M. Maxey for assistance in collecting common tern eggs and American kestrel eggs, and Karen M. Eisenreich for bone length measurements. We also thank Sandra Schultz for assistance with the oxidative stress assays. Ellen Harvey and Mark J. La Guardia performed the analysis of egg and dosing solutions for halogenated target compounds. This work was funded in part by the San Francisco Estuary Institute (Contract No. 869).

References

Chen D, Hale RC. 2010. A global review of polybrominated diphenyl ether flame retardants contamination in birds. *Environ Internat* 36:800-811.

Chen D, Zhang X, Mai B, Song J, Sun Q, Luo Y, Luo X, Zheng EY, Hale RC. 2009. Polychlorinated biphenyls and organochlorine pesticides in various bird species from northern China. *Environ Poll* 157:2023-2029.

Chen D, La Guardia MJ, Harvey E, Amaral M, Wohlfort K, Hale RC. 2008. Polybrominated diphenyl ethers in peregrine falcon (*Falco peregrinus*) from the northeastern U.S. *Environ Sci Tech* 42:7594-7600.

Chipley RM, Fenwick GH, Parr MJ, Pashley DN. 2003. The American Bird Conservancy Guide to the 500 Most Important Bird Areas in the United States. New York: Random House. 518pp.

Fernie KJ, Mayne G, Shutt LJ, Pekarick C, Grasman KA, Letcher RJ, Droullard KG, 2005a. Evidence of immunomodulation in nestling American kestrels (*Falco sparverius*) exposed to environmentally relevant PBDEs. *Environ Pollut* 138:485-493.

Fernie KJ, Shutt LJ, Mayne G, Hoffman DJ, Letcher RJ, Droullard KG, Richie IJ. 2005b. Exposure to polybrominated diphenyl ethers (PBDEs): changes in thyroid, vitamin A, glutathione homeostasis, and oxidative stress in American kestrels (*Falco sparverius*). *Toxicol Sci* 88:375-383.

Fernie KJ, Shutt LJ, Ritchie JL, Letcher RJ, Droullard KG, Bird DM. 2006. Changes in the growth, but not the survival, of American kestrels (*Falco sparverius*) exposed to environmentally relevant polybrominated diphenyl ethers. *J Toxicol Environ Health Part A* 69:1541-1554.

Fernie KJ, Shutt JL, Letcher RJ, Ritchie JI, Sullivan K, Bird DM. 2008. Changes in reproductive courtship behaviors of adult American kestrels (*Falco sparverius*) exposed to environmentally relevant levels of the polybrominated diphenyl ether mixture, DE-71. *Toxicol Sci* 102:171-8.

Fernie KJ, Shutt JL, Letcher RJ, Ritchie JI, Bird DM. 2009. Environmentally relevant concentrations of DE-71 and HBCD alter eggshell thickness and reproductive success of American kestrels. *Environ Sci Tech* 43:2124-2130.

Fox GA, Gilman AP, Peakall DB, Anderka FW. 1978. Behavioral abnormalities of nestling Lake Ontario herring gulls. *J Wildl Manage* 42:477-483.

Fowles JR, Fairbrother A, Baecher-Steppan L, Kerkvliet NI. 1994. Immunologic and endocrine effects of the flame-retardant pentabromodiphenyl ether (DE-71) in C57BL/6J mice. *Toxicol* 86:49–61.

French JB Jr. Nisbet ICT, Schwabl H. 2001. Maternal steroids and contaminants in common tern eggs: a mechanism of endocrine disruption? *Comp Biochem Physiol* 128C: 91-93.

Gauthier LT, Hebert CE, Weseloh DV, Letcher RJ. 2008. Dramatic changes in the temporal trends of polybrominated diphenyl ethers (PBDEs) in herring gull eggs from the Laurentian Great Lakes: 1982-2006. *Environ Sci Tech* 42:1524-1530.

Giordano G, Kavanagh TJ, Costa LG. 2008. Neurotoxicity of a polybrominated diphenyl ether mixture (DE-71) in mouse neurons and astrocytes is modulated by intracellular glutathione levels. *Toxicol Appl Pharmacol* 232: 161-168.

Golden NH, Rattner BA. 2003. Ranking terrestrial vertebrate species for utility in biomonitoring and vulnerability to environmental contaminants. *Rev Environ Contam Toxicol* 176:67-136.

Hallgren S, Sinjari T, Hakansson H. 2001. Effects of polybrominated diphenyl ethers (PBDEs)and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in ratsand mice. *Arch Toxicol* 75:200–2008.

Hallgren S, Darnerud PO. 2002. Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats—Testing interactions and mechanisms for thyroid hormone effects. *Toxicology* 177:227–243.

Hays H, LeCroy M. 1971. Field criteria for determining incubation stage in eggs of the common tern. *Wilson Bull* 83:425-429.

Heinz GH, Stebbins KR, Klimstra JD, Hoffman DJ. 2009a. A simplified method for correcting contaminant concentrations in eggs for moisture loss. *Environ Toxicol Chem* 28:1425-1428.

Heinz GH, Hoffman DJ, Klimstra JD, Stebbin KR, Konrad SL, Erwin CA. 2009b. Species differences in the sensitivity of avian embryos to methylmercury. *Arch Environ Contam Toxicol* 56:129-138.

Henny CJ, Kaisr JL, Grove RA, Letcher RJ. 2009. Polybrominated diphenyl ether flame retardants in eggs may reduce reproductive success of ospreys in Oregon and Washington, USA. *Ecotoxicology* 18:802-813.

Henny CJ, Grove RA, Kaiser JL, Johnson BL, Furl CV, Letcher RJ. 2011. Wastewater dilution index partially explains observed polybrominated diphenyl ether flame retardant concentrations in osprey eggs from Columbia River Basin, 2008-2009. *Ecotoxicology* 20: 682-697.

Hoffman DJ, Melancon MJ, Klein PN, Eisemann JD, Spann JW. 1998. Comparative developmental toxicity of planar polychlorinated biphenyl congeners in chickens, American kestrels, and common terns. *Environ Toxicol Chem* 17:747-757.

Hoffman DJ, Spalding MG, Frederick PC. 2005. Subchronic effects of methylmercury on plasma and organ biochemistries in great egret nestlings. *Environ Toxicol Chem* 24: 3078-3084.

Karnofsky DA. 1965. The chick embryo in drug screening: Survey of teratological effects observed in the 4-day-old chick embryo. In Wilson JG, Warkany JK, eds, *Teratology: Principles and Techniques.* University of Chicago Press, Chicago, IL, USA, pp. 194-213.

Klimstra JD, Stebbins KR, Heinz GH, Hoffman DJ, Kondrad SR. 2009. Factors related to the artificial incubation of wild bird eggs. *Avian Biol Res* 2:121-131.

La Guardia MJ, Hale RC, Harvey E. 2006. Detailed polybrominated diphenyl ether (PBDE) congener composition of the widely used Penta-, Octa-, and Deca-PBDEs technical flame-retardant mixtures. *Environ Sci Technol* 40:6247-6254.

Lu SC. 1999. Regulation of hepatic glutathione synthesis: current concepts and controversies. *FASEB J* 13:1169-1183.
Marnett LJ. 1999. Lipid peroxidation-DNA damage by malondialdehyde. *Mutation Research*. 8: 83-95.

Marteinson SC, Bird DM, Shutt JL, Letcher RJ, Ritchie IJ, Fernie KJ. 2010. Multi-generational effects of polybrominated diphenylethers exposure: embryonic exposure of male American kestrels (*Falco sparverius*) to DE-71 alters reproductive success and behaviors. *Environ Toxicol Chem* 29:1740-1747.

McKernan, MA, Rattner BA, Hale RC, Ottinger MA. 2007. Egg incubation position affects toxicity of air cell administered polychlorinated biphenyl 126 (3,3',4,4',5-pentachlorobiphenyl) in chicken (*Gallus gallus*) embryos. *Environ Toxicol Chem* 26:2724-2727.

McKernan MA, Rattner BA, Hale RC, Ottinger MA. 2009. Toxicity of polybrominated diphenyl ethers (DE-71) in chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), and American kestrel (*Falco sparverius*) embryos and hatchlings. *Environ Toxicol Chem* 28:1007-1017.

McKernan MA, Rattner BA, Hatfield J, Hale RC, Ottinger MA. 2010. Uptake and biotransformation of polybrominated diphenyl ethers DE-71 and DE-79 in chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), American kestrel (*Falco sparverius*) and black-crowned night-heron (*Nycticorax nycticorax*) eggs. *Chemosphere* 79:100-109.

Monaghan P, Metcalfe NB, Torres, R. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecol Lett* 12:75-92.

Norstrom RJ, Simon M, Moisey J, Wakeford B, Weseloh DV. 2002. Geographical distribution (2000) and temporal trends (1981-2000) of brominated diphenyl ethers in Great Lakes herring gull eggs. *Environ Sci Technol* 36:4783-4789.

Potter KE, Watts BD, La Guardia MJ, Harvey EP, Hale RC. 2009. Polybrominated diphenyl ether flame retardants in Chesapeake Bay region USA, peregrine falcon (*Falco peregrines*) eggs: urban/rural trends. *Environ Toxicol Chem* 28: 973-981.

Rahn H, Ar A. 1975. The avian egg: Incubation time and water loss. *Condor* 76:147-152.

Rattner BA, Eisenreich KM, Golden NH, McKernan MA, Hothem RL, Custer TW. 2005. Retrospective ecotoxicological data and current information needs for terrestrial vertebrates residing in coastal habitat of the United States. *Arch Environ Contam Toxicol* 49:257-265.

Rattner BA, McGowan PC, Golden NH, Hatfield JS, Toschik PC, Lukei RF Jr, Hale RC, Schmitz-Afonso I, Rice CP. 2004. Contaminant exposure and reproductive success of ospreys (*Pandion haliaetus*) nesting in Chesapeake Bay regions of concern. *Arch Environ Contam Toxicol* 47:126-140.

Rattner BA, McGowan PC. 2007. Potential hazards of environmental contaminants to avifauna residing the Chesapeake Bay estuary. *Waterbirds* 30: 63-81.

Richardson VM, Staskal DF, Ross DG, Dilberto JJ, DeVito MJ, Birnbaum LS. 2008. Possible mechanisms of thyroid hormone disruption in mice by BDE-47, a major polybrominated diphenyl ether congener. *Toxicol Appl Pharmocol* 226:244–250.

She J, Holden A, Adelsbach TL, Tanner M, Schwarzbach SE. 2008. Concentrations and timetrends of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) in aquatic bird eggs from San Francisco Bay, CA 2000-2003. *Chemosphere* 73:S201 S209.

Storey KB. 1996. Oxidative stress: animal adaptations in nature. *Braz J Med Biol Res* 29: 1715-33.

[US EPA] US Environmental Protection Agency. 2011. Flame-retardant alternatives for decaBDE partnership. http://www.epa.gov/dfe/pubs/projects/decaBDE/index.htm.

Wiemeyer SN, Clark DR, Spann JW, Belisle AA, Bunck CM. 2001. Dicofol residues in eggs and carcasses of captive American kestrels. *Environ Toxicol Chem* 20:2848-2851.

Xie X. Wu Y, Zhu M, Zhange Y-k. Wang X. 2011. Hydroxyl radical generation and oxidative stress in earthworms (*Eisenia fetida*) exposed to decabromodiphenyl ether (BDE-209). *Ecotoxicology* 20:993-999.

Zhou T, Ross DG, de Vito MJ, Crofton KM. 2001. Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol Sci* 61:76–82.