Lessons Learned, Challenges, and Opportunities:
The US Endocrine Disruptor Screening Program

Poster Titles and Abstract Booklet

Poster Session
April 23, 2013; 5-7 pm

Assembly and printing of Poster Abstract booklet provided by:

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1.

Permethrin Does Not Have Endocrine Disrupting Properties as Evaluated by the U.S. EPA's Endocrine Disruptor Screening Program (EDSP) *In Vivo* Assays

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Permethrin, a pyrethroid insecticide, was evaluated for potential endocrine activity in 3 of 4 *in vivo* US EPA EDSP assays. The maximum tolerated dose (MTD) of permethrin used in these assays was selected based on the results of dose range finding studies. Sprague Dawley (SD) rats were administered permethrin in corn oil by oral gavage for 10 (Hershberger Bioassay, OPPTS 890.1600, castrated model), 21/22 (Female Pubertal Assay, OPPTS 890.1450), or 31/32 (Male Pubertal Assay, OPPTS 890.1500) days based on daily body weights. The Hershberger Bioassay, which evaluated agonist and antagonist androgenic activity, was negative; no significant changes in any androgen-dependent tissue weights up to the MTD of 120 mg/kg/day were observed. The male pubertal assay, administered a MTD of 120 mg/kg/day, showed no effects on male pubertal development with no differences observed in reproductive organ weights, the day of preputial separation, or serum testosterone (T) levels compared to controls. Liver weight was significantly increased, along with a decrease in thyroxine (T_4) with a corresponding increase in thyroid stimulating hormone (TSH) compared to controls. No changes were observed the thyroid gland weights or histopathology. The female pubertal assay, administered a MTD of 150 mg/kg/day, showed no effects on female pubertal development with no differences observed in the day of vaginal opening, estrus onset, cyclicity, or reproductive organ weights. The liver weights were significantly increased, thyroid gland weights significantly decreased, and serum TSH concentrations were increased with a corresponding decrease in serum T_4 following administration permethrin; however, the thyroid gland showed no histopathological changes. In summary, permethrin did not show androgen or estrogen activity in these assays, and the effects observed in the thyroid likely are secondary to liver enzyme induction, a phenomenon known to occur following repeated dosing with permethrin, and not a reflection of a direct effect on the thyroid.

2.

Integrated Laboratory Systems Historical Control Data from the Endocrine Disruptor Screening Program (EDSP) Tier 1 Uterotrophic and Hershberger Assays

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The U.S. EPA Endocrine Disruptor Screening Program (EDSP) includes four *in vivo* mammalian assays to screen test substances for potential disruption of the estrogen, androgen, or thyroid hormone pathways. The Uterotrophic Assay screens for potential estrogenic activity and can be performed in immature intact females or ovariectomized adult rats. Animals are administered the test substance for three days, and at termination the wet and blotted uterine weights are obtained. A positive estrogenic response in the assay is a significant increase in the uterine weights. The performance criteria (vehicle control blotted uterine weights as a percent of body weight) were met in all studies conducted in either animal model. The Hershberger Bioassay screens for both potential androgenic and anti-androgenic activity in the adult male castrated rat. Animals are administered test substance for ten consecutive days and at termination five androgen-dependent tissues (glans penis, ventral prostate, seminal vesicle, Cowper's gland, and levator ani plus bulbocavernosus muscle) are excised and weighed. A positive response in the agonist assay or antagonist assay is a significant increase or decrease, respectively, in at least two tissue weights compared to controls. The performance criteria (no more than 2 of 10 tissues at or below coefficient of variation in control and high dose groups) were met in all assays. We demonstrate that the performance criteria in the Uterotrophic and Hershberger Assays are attainable and repeatable in our laboratory, and multiple vehicles may be used to administer the test substance without concern of meeting the performance criteria.

3.

Integrated Laboratory Systems Historical Control Data from the Endocrine Disruptor Screening Program (EDSP) Tier 1 Female Pubertal Assay

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The U.S. EPA Endocrine Disruptor Screening Program (EDSP) includes four *in vivo* mammalian assays to screen test substances for potential disruption of the estrogen, androgen, or thyroid hormone pathways. The Female Pubertal Development and Thyroid Function Assay (OPPTS 890.1450) screen for disruption of the estrogen, hypothalamic-pituitary-gonadal axis, and thyroid hormone pathways. This assay is conducted in the female Sprague Dawley rat with daily test substance administration from postnatal (PND) 22 to 42/43. Daily body weights, clinical observations, and the day of vaginal opening and estrous cyclicity were evaluated. Two hours after the final dose administration, animals are humanely euthanized and blood and tissues are collected. Changes in tissue weights, serum hormone concentrations (thyroxine and thyroid stimulating hormone) and clinical chemistry endpoints, and histopathological evaluation of selected tissues (thyroid, ovaries, uterus, kidney) were evaluated. Here we demonstrate our ability to meet the performance criteria set forth by the test guidelines for data collected; including final body weight, day age and body weight at vaginal opening, tissue weights (liver, kidneys, adrenal glands, pituitary, ovaries, thyroid, and wet and blotted uterus), and thyroxine and thyroid stimulating hormone levels concentrations from multiple vehicles. We demonstrate that the performance criteria in the female pubertal assay are attainable and repeatable in our laboratory, and multiple vehicles may be used to administer the test substance without concern of meeting the performance criteria.

4.

Integrated Laboratory Systems Historical Control Data from the Endocrine Disruptor Screening Program (EDSP) Tier 1 Male Pubertal Assay

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The U.S. EPA Endocrine Disruptor Screening Program (EDSP) includes four in *vivo* mammalian assays to screen test substances for potential disruption of the estrogen, androgen, or thyroid hormone pathways. The Male Pubertal Development and Thyroid Function Assay (OPPTS 890.1500) screen for disruption of the androgen and thyroid hormone pathways, and hypothalamic-pituitary-gonadal axis. This assay is conducted in the male Sprague-Dawley rat from postnatal (PND) 23 to 53/54, and evaluates the day of preputial separation until complete. Two hours after final dose administration, animals are humanely euthanized and blood and tissues are collected. Changes in tissue weights, serum hormone concentrations (thyroxine, thyroid stimulating hormone, and testosterone) and clinical chemistry endpoints, and histopathological evaluation of selected tissues (thyroid, testis, epididymis, kidney) were evaluated. Here we demonstrate our ability to meet the performance criteria set forth by the guidelines for data collected including final body weight, age and weight at day of preputial separation, tissue weights (liver, kidney, adrenal glands, pituitary, testes, epididymides, dorsolateral and ventral prostate, seminal vesicle, levator ani plus bulbocavernosus muscle), from multiple vehicles. We demonstrate that the endpoints and performance criteria in the male pubertal assay are repeatable in our laboratory, almost all performance criteria are attainable, and multiple vehicles may be used to administer the test substance without concern of meeting the performance criteria.

5.

Control Data from the Three Combined Male and Female Rat Pubertal Assays (OCSPP 870.1500 and 870.1450) plus Two Validation Studies

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F_1 pups were weaned on PND 21 and assigned to groups (15/sex/group and housed 3/sex/cage). The pups were dosed orally once daily from PND 22 to 42 (females) or from PND 23 to 53 (males). Clinical observations and bodyweight were recorded daily. Feed consumption was recorded every 3 days. Sexual maturation (vaginal opening and preputial separation) was evaluated daily from initiation of dosing until acquisition. Upon vaginal opening, estrous smears were taken and evaluated for day of first estrus, number of cycles, cycle length, and cycle pattern. Necropsies were performed 2 hours after final dose. Blood was collected for hormone analyses (thyroxine, testosterone (males) and thyroid stimulating hormone), and selected tissues (thyroid, liver, kidneys, pituitary and adrenals for all pups; testis, epididymides, ventral prostate, dorsolateral prostate, seminal vesicle {with coagulating glands and fluid}, levator ani/bulbocavernosus muscles for males; and uterus and ovaries for females) were harvested, weighed, and selected tissues were paraffin-embedded, sectioned, H&E stained and evaluated for pathologic abnormalities. The follicular epithelial height and colloid area from thyroid sections were determined. Ovarian follicle count using the PCNA stain was performed.
Control individual and data from three combined male and female pubertal plus two validation studies are presented (~75 pups/sex/endpoint) and compared with the EPA's Performance Criteria. The use of a Combined Male and Female Pubertal Design versus the stand-alone Male Pubertal Study and Female Pubertal Study will also be discussed in relation to the "3 R's" reduction of animal usage and its associated cost saving.

6.

A Comparative Assessment of Four *in Vivo* Assays Included in the Tier 1 Screening Battery for the Endocrine Disruptor Screening Program (EDSP) in the Sprague Dawley Rat

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In 2009, the U.S. Environmental Protection Agency published the EDSP Tier 1 battery of assays, study guidelines for conducting these assays, and the first list of around 65 chemicals with test orders. Between 2010-2012, WIL Research conducted over 90 studies intended for submission under the EDSP. Here, we present a comparative assessment of data derived from four of the rodent *in vivo* assays included in the Tier 1 Screening Battery – Uterotrophic, Hershberger and the male and female Pubertal assays in the Sprague Dawley rat. Parameters presented include adult male and female body, and reproductive organ weights, pup growth parameters, developmental landmarks (vaginal patency and balanopreputial separation), female estrous cyclicity, serum chemistry, serum hormones (T4, TSH, and Testosterone), organ weights, including accessory and primary reproductive organs and histopathological findings. Interpretation of data obtained during the conduct of developmental and reproductive toxicity studies typically involves the widespread use of historical control datasets as a basis for interpretation. Due to the recent introduction of the pubertal assay guidelines and the infrequent use of the Uterotrophic and Hershberger assays prior to their inclusion in the Tier 1 Battery, relatively little historical control data was available for reference during these studies. This presentation is an effort to mine the extensive data obtained from over 90 studies to identify discernible trends in pubertal development, endocrine physiology and alterations in reproductive organ weights (both adult and juvenile) following exposure to a variety of chemicals tested under the Endocrine Disruptor Screening Program.

7.

Histologic Features of Prepubertal and Pubertal Ovarian Development in Sprague-Dawley Rats

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In response to growing concerns that environmental chemicals may have adverse effects on human health by altering the endocrine system, the Endocrine Disruptor Screening Program (EDSP), under the auspices of the United States Environmental Protection Agency (US EPA), recently instituted male and female pubertal assays as part of the Tier I battery of tests for evaluating compounds for possible adverse effects on the developing endocrine system. The female pubertal assay requires oral gavage of female rats with test substance from postnatal day (PND) 22 through PND 42 or 43, the period for development of sexual maturity in the rat. Microscopic examination of the ovary at PND 42 or 43 is a critical component of the female pubertal assay. While certain landmarks in female reproductive development are published, such as onset of estrus (PND 29–38), vaginal patency (PND 33), and sexual maturity (PND 49), little is published on the microscopic appearance of the ovary during prepubertal and pubertal development. In this study, reproductive tissues from three female Sprague Dawley rats were collected each day from PND 20 through PND 50, such that tissues from a total of ninety-three rats were collected throughout the pre-pubertal and pubertal period. Ovaries were formalin-fixed, trimmed, paraffin-embedded, sectioned at 5 μm thickness, and examined microscopically. The major histologic features of the ovaries throughout this critical period were described in detail. This information will help pathologists interpret findings observed in female pubertal assays.

8.

Lack of Estrogenic Potential of Monomers Utilized in Eastman Tritan™ Copolyster

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Eastman Chemical Company manufactures several polyester plastics including Tritan™ copolyester manufactured utilizing three monomers, di-methylterephthalate (DMT), 1,4-cyclohexanediemthanol (CHDM), and 2,2,4,4-tetramethyl-1,3-cyclobutanediol (TMCD) in various ratios. Tritan™ is utilized in many consumer products and has been approved for use by various governmental regulatory agencies for use in both food contact and medical device applications. As with most polymers, the monomers along with the high molecular weight oligomers whose toxicity is best represented by the monomers, make up the predominate chemicals available for leaching into the environment and foods. In light of the high level of public concern about the presence of estrogenic activity ascribed to certain plastic monomers and chemicals in the environment, Tritan's™ monomers were evaluated using a comprehensive battery of *in vitro* and *in vivo* techniques to understand whether they might pose such a concern. Specifically, these monomers were assessed for estrogen receptor binding activity potential using both QSAR and a fluorescent labeled ligand displacement assay. In addition, the monomers were assessed for their ability to activate estrogen receptors using T47D-KBluc cells and in a bioluminescent yeast-based detection method. Estrogenic activity potential was also screened for through the conduct of an uterotrophic assay. Results from all these studies were uniformly negative and when coupled with other *in vivo* data developed to assess systemic toxicity, and developmental and reproductive toxicity the preponderance of all evidence clearly indicates that these monomers, and potentially the oligomers which contain similar functional groups, do not pose an estrogenic risk to humans or the environment.

9.

Polyester Monomers Utilized by Eastman Chemical Company Lack Ability to Bind and Activate both Estrogenic and Androgenic Receptors

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Eastman Chemical Company is a manufacturer of several polyester plastics that have been approved by various governmental regulatory agencies for use in both food contact and medical device applications. As with most polymers, monomers and high molecular weight oligomers, whose toxicity is best represented by the monomers, make up the predominate chemicals within a polymer available for migrating into the environment and foods. In light of recent public attention over the presence of endocrine active compounds in the environment from a variety of sources, including plastics, studies were conducted to understand whether the monomers utilized in the manufacture of many of Eastman's plastics possessed the ability to bind to and activate either estrogen and androgen receptors. Assessment for their binding potential to estrogen and androgen receptors was evaluated by QSAR and via fluorescent labeled ligand displacement assay. In addition to understanding binding affinity potentials, the monomers were assessed for their ability to activate estrogen receptors usingT47D-KBluc cells and androgenic receptors using MDA-KB2 cells. Quantification of the response in both assays is based on the activation and expression of a promoter linked luciferase gene. Results of all studies clearly demonstrate that there was a lack of an interaction with these monomers to the estrogen or androgen receptors in the absences of cytotoxicity. When these *in vitro* results are viewed in the context of *in vivo* systemic toxicity studies; the evidence, in total, clearly indicates that these monomers would be unlikely to pose an estrogenic or androgenic risk to humans or the environment.

10.

Mapping Pathways of Toxicity of Endocrine Disruption: Metabolomic Characterization of the Effects of Estradiol in MCF-7 Cell Line

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The NIH transformative project "Mapping the Human Toxome by Systems Toxicology" represents an initiative promoting the implementation of a new toxicity testing paradigm as envisioned by TT21C. Our objective is to employ high-throughput liquid chromatography mass spectrometry based metabolomics analysis, in combination with other omics, to map pathways affected by endocrine disrupting compounds. The poster will focus on the metabolomic characterization of the effects of Estradiol (E2) in MCF-7 human breast cancer cell Lines. E2 is the most potent naturally occurring estrogen. Its effects on cell function during development and in adults in a variety of tissues are mediated through estrogen receptors located in the nucleus, cytoplasm, mitochondria and at the cell membrane. Concurrent cell growth and gene expression studies are also being carried out. Cells were exposed to E2 at different concentrations and times. Stimulation of cell growth was observed at 0.1nM in association with changes in the metabolome at 6- and 24-hours. A time course study at 100nM E2 revealed that some metabolites were significantly altered at various time points. The metabolites and pathways most affected included metabolites from cellular energy-related pathways (e.g., Malate, Fumarate, Puruvate) and amino acid synthesis pathways (e.g., Arginine, Valine, Ornithine, Leucine/Isoleucine, 2-Methylmalate). Further investigation is underway and should give more insight into the identification of metabolism pathways affected by E2 and endocrine disruptors.

11.

Creating in vitro test methods for endocrine disruptor risk assessment: Assay development

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Endocrine disruptor test programs in the US are moving forward to identify active compounds and prioritize them for subsequent in-life toxicity studies. Our focus is on developing *in vitro* tests for cellular responses to endocrine active substances that will be sufficient for health risk assessment without moving on to in-life toxicity tests.
We have begun a research effort for the estrogen receptor (ER) pathway to: 1) Map signaling pathways for estrogen mediated uterine epithelial cell proliferation, 2) Define the dose-response for perturbation of estrogen signaling by xenobiotics, and 3) Develop computational models to predict chemical effect on uterine epithelium. The first phase develops a cell-based assay in human Ishikawa endometrial cell line expressing the three major ERs: ESR1, ESR2 and GPER. The dose-responses to treatment with 17α-ethynyl estradiol (EE) were examined over a 6 day period for cell proliferation and induction of known ESR1 targets, including alkaline phosphatase (ALP), proliferation associated gene GREB1 and progesterone receptor (PGR). Ishikawa cells showed an increased proliferation and up-regulation of ESR1 target genes upon EE treatment. We thus determined doses and times at which transcriptomic analyses are being performed to study estrogen activated pathways. Moreover, in order to identify receptor-specific activated pathways, cells were also treated with selective receptor agonists PPT, DPN, and G1 targeting ESR1, ESR2 and GPER, respectively. PPT increased cell proliferation and ALP activity similar to EE, while DPN and G1 had no effects on tested outputs. Finally, we develop computational tools to dynamically analyze genomic data generated by the cell-based assays.

12.

Adaptation of the BG1Luc Estrogen Receptor Transactivation Test Method to qHTS: Comparison of Results from Both Methods

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In 2011, the Interagency Center for the Evaluation of Alternative Toxicological Methods nominated the BG1Luc estrogen receptor (ER) transactivation (TA) test method (BG1Luc ER TA) to Tox21 to be adapted into a quantitative high-throughput screening (qHTS) format. The Tox21 collaboration, an effort by the National Toxicology Program, NIH Chemical Genomics Center, Environmental Protection Agency, and Food and Drug Administration, was formed to advance toxicity testing by shifting from traditional *in vivo* tests to *in vitro* methods. A major goal of Tox21 is to prioritize chemicals for in-depth toxicity testing. One approach for prioritization is to use qHTS cell- and biochemical-based assays to construct concentration–response curves for thousands of chemicals. The Tox21 consortium adapted the BG1Luc ER TA method to a qHTS format. Data were generated for approximately 10,000 chemicals in both the agonist and antagonist versions of the qHTS assay. Seventy-six chemicals were tested in both the BG1Luc ER TA manual and qHTS methods. These data were used to evaluate the degree to which classifications of test chemicals in the manual and qHTS methods matched the classifications for performance standards reference substances (accuracy) and the degree to which the classifications were identical between the two methods (concordance). Agonist and antagonist methods produced 97 to 100% accuracy and 93 to 96% concordance, respectively, demonstrating that the performance of the qHTS format is comparable to the validated BG1Luc ER TA. (ILS staff supported by NIEHS contract N01-ES 35504.)

13.

Development of a High-Throughput Thyroperoxidase Inhibition Assay for Thyroid-Disruptor Screening

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Thyroperoxidase (TPO), the catalyst for thyroid hormone (TH) synthesis, is a target for thyroid-disruptors, including methimazole (MMI), isoflavones, benzophenone-2, and malachite green; however, no medium- to high-throughput screening methods for TPO inhibition are available. To adapt the low-throughput guaiacol oxidation assay for TPO inhibition to chemical screening, we replaced guaiacol with a fluorescent peroxidase substrate in a rat thyroid-based assay and developed an *in vitro* human TPO model. We tested the hypothesis that use of a peroxidase substrate (Amplex UltraRed, AUR, LifeTech) in 96- or 384-well plate formats with automated reagent delivery could increase the TPO assay throughput with thyroid microsomes. The IC50s of MMI-induced TPO inhibition in the 96- and 384-well plate formats were 0.093 and 0.025 µM, respectively, compared to 2.20 µM in the guaiacol-based 96-well format. The AUR signal was stable from 30-120 min after initiation. The dynamic range of the AUR-TPO assay with MMI was 11- to 18-fold using 96- or 384-well formats, with Z' scores of 0.82 to 0.93. A 21 chemical training set, comprised of predicted positive and negative chemicals, was used to validate the assay for screening, and demonstrated a 100% true positive rate and a 0% false negative rate. One limitation of this model is the availability of human thyroid microsomes. Preliminary work has demonstrated that lentiviral-transduction of a human cell line (HEK293T) with recombinant TPO could also be used to test for human TPO inhibition. TPO activity measured by the AUR assay in cell-based microsomes was inhibited in a dose-responsive manner, with maximal inhibition at 100 µM MMI. This approach to improving and developing high-throughput assays for thyroid-disruptor screening demonstrates the feasibility of screening 100s to 1000s of chemicals for TPO inhibition, drastically reduces the need for animal tissue, and highlights the potential to completely replace animal tissue with cell-derived protein. *This abstract does not necessarily reflect the policy of the US EPA.*

14.

Differences in the age- and time-dependent effects of pyriproxyfen on fecundity and sex determination in adult and juvenile *Daphnia magna*

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Pyriproxyfen is a juvenile hormone analog that induces male production in a normally all female, parthenogenically reproducing *Daphnia* population. The induction of male production can significantly reduce reproduction and reduce population size. In this study, we investigated the impact of pyriproxyfen on *Daphnia magna* fecundity, male production, and development in juvenile and adult daphnids. First, ten day old adult daphnids were exposed to pyriproxyfen for approximately 10-12 days or until they had 4 broods, and the number and sex of the offspring determined. Pyriproxyfen reduces overall fecundity and increases male production in a concentration-dependent fashion with an EC50 of 156 pM (50.24 ng/L). Next, we examined the adverse reproductive effects of acute and chronic 155 and 310 pM pyriproxyfen exposures (2, 4, 8, or 12 day exposures) on juvenile (3-days old) and reproductively mature *Daphnia* (10-days old). Results indicate that longer pyriproxyfen exposures (8-12 days) extend male production and decrease reproduction; while daphnids exposed for only 2-4 days recover and produce a relatively normal abundance of neonates. In addition, juvenile daphnids are also very sensitive to pyriproxyfen, but the primary effect on juvenile daphnids is reduced reproduction and protracted development not male

production. Taken together, continued use of pyriproxyfen around water bodies needs due caution because of its potential adverse effects with significant developmental delays and male production compounded by prolonged exposure.

15.

Can HR96 Activation or Inhibition Alter Toxicity in *Daphnia?*

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Daphnia are planktonic crustaceans widely used for toxicology testing. HR96 is a promiscuous endo- and xenobiotic nuclear receptor thought to induce phase I – III detoxification enzymes, and an ortholog of CAR/PXR found in vertebrates. In *Daphnia*, HR96 is activated by chemicals such as atrazine, chlorpyrifos, pyriproxyfen, estradiol, and linoleic acid (LA) (n-6 fatty acid). Triclosan and the n-3 fatty acid, DHA inhibit HR96. We hypothesize that inhibitors of HR96 could potentially block the protective responses of HR96 and in turn cause synergistic toxicity. We have performed acute toxicity tests with triclosan, atrazine, docosahexaenoic acid (DHA) (n-3), and linoleic acid (n-6). LC50s to the polyunsaturated fatty acids are typically about 10 µM, with DHA showing the greatest toxicity. Triclosan and atrazine have LC50's of 0.84 and 78 µM, respectively. Acute mixture toxicity tests were performed using an inhibitor (DHA or triclosan) and an activator (LA or atrazine) in each assay. Surprisingly, results demonstrated that atrazine decreases the toxicity of triclosan and DHA, presumably by activating HR96 and inducing protective enzymes. Twenty–Forty µM atrazine provided protection from triclosan and DHA. However, the weak HR96 activator, LA did not provide protection from triclosan or DHA. We hypothesize that atrazine is protective because it activates HR96 and induces phase I-III enzymes, providing a potential mechanism by which some xenobiotics and dietary components could alter an individual's sensitivity to specific chemicals. Future studies will involve examining the changes in gene expression associated with atrazine, and determining how atrazine alters triclosan metabolism and clearance.

16.

Validation of the Amphibian Metamorphosis Assay for Potential Endocrine Disrupting Chemicals with *Xenopus laevis*

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The European Union Plant Protection Products Regulation (PPPR 1107/2009) identifies a requirement for consideration on whether a substance has potential endocrine disrupting effects in aquatic non-target organisms. The requirements refer to screening assays for ecotoxicological endocrine-disrupting potential. We describe in detail our experience in the establishment and validation of the amphibian metamorphosis assay (OECD 231; OPPTS 890.1100) with *Xenopus laevis*.
In this method, in order to satisfy rearing phase validity criteria, conditions necessary for development from fertilisation to stage 51 as defined by Nieuwkoop and Faber (1994) were established, and individuals selected for the exposure phase. To establish the assay, 400 tadpoles were exposed to the three reference substances, thyroxine (T_4) which produces stimulatory effects on the normal function of the hypothalamic-pituitary-thyroid (HPT) axis, sodium perchlorate (which retards development) and iopanoic acid (which affects hind limb development) and levels of each were verified using appropriate analytical methods. At Day 7, 80 randomly selected individuals at each level were removed and assessed (body weight, developmental stage, hind limb and snout to vent length). Exposure continued for a further two weeks, with study termination on Day 21 when all remaining individuals were assessed as on Day 7. Following developmental stage matching, 80 individuals were selected for thyroid removal and histopathological analysis.

We found that our results were similar to the ring test results published by the OECD (Series on Testing and Assessment Document Number 77) and make a number of observations on methodology that may improve the reproducibility of these assays.

17.

Glyphosate: Fish Short-Term Reproduction Assay (FSTRA) with the Fathead Minnow (Pimephales promelas)

Holmes, K., Schneider, S., Krueger, H., Claude, J., Ross, T., Palmer, S., Springer, T., Jaber, M., Gallagher, S., Leopold, A.
BASF Corporation; Wildlife International Ltd., Easton, MD

A fish short-term reproduction assay was conducted by Wildlife International, Ltd. for the Joint Glyphosate Task Force at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland from December 21, 2011 to January 11, 2012. The objective of this assay was to determine if glyphosate might impact the hypothalamus-pituitary-gonadal (HPG) endocrine axis resulting in the disruption of reproduction in fish. Breeding groups of fathead minnows (*Pimephales promelas*) were exposed to glyphosate under flow-through conditions at mean measured concentrations of 0.046, 0.23, 1.2, 6.2 and 33 mg a.e./L for 21 days. Endpoints that were evaluated for endocrine disruption of the reproductive system included fecundity, fertility, secondary sex characteristics (including tubercle and fatpad scores), gonadosomatic index (GSI), histopathology of gonads, as well as plasma vitellogenin. Other endpoints included survival, general observations of health, weight, and length. There were no apparent effects on survival, growth, reproduction, secondary sex characteristics, GSI, VTG or gonad histopathology in male or female fish exposed to glyphosate for 21 days. Based on the endpoints evaluated, glyphosate does not appear to impact the function of the hypothalamus-pituitary-gonadal (HPG) endocrine axis in fathead minnows.

18.

Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances

Holmes, K., Schneider S., Claude J., Krueger, H., Springer, T., Ross, T., Gallagher, S., Palmer, S., Jaber, M.
BASF Corporation; Wildlife International Ltd., Easton, MD

An amphibian metamorphosis assay was conducted by Wildlife International, Ltd. for the Joint Glyphosate Task Force at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland from October 24, 2011 to November 14, 2011. African clawed frog (*Xenopus laevis*) tadpoles were exposed to glyphosate at mean measured concentrations of 0.13, 0.79, 4.3, 20 and 90 mg a.e./L for 21 days under flow through conditions. Endpoints that were evaluated to determine if the test substance impacted the HPT axis included survival, gross morphological abnormalities, developmental stage, wet weight, body length, snout-to-vent length, normalized hind-limb length, and thyroid gland histology. There were no treatment-related effects on survival, stage, or normalized hind limb length during the 21-day test. Histopathologic analysis showed no treatment-related changes in the thyroid glands of *Xenopus laevis* tadpoles when compared to negative control animals. There was a slight increase in wet weight in the 90 mg a.i./L treatment group and in snout-to-vent length in the 4.3 and 90 mg a.e./L treatment groups at the end of the 21-day test. However, since there were no effects observed on normalized hind-limb length, stage, or thyroid histology, these increases are not indicative of a thyroid effect. Glyphosate was not found to interfere with the normal function of the hypothalamus-pituitary-thyroid axis of African clawed frog tadpoles in this study.

19.

The Evaluation of Control Performance in the Amphibian Metamorphosis Assay Against the OPPTS 890.1100

Michael Lee, Lee E. Sayers, Ronald C. Biever
Smithers Viscient, Wareham, MA

The United States Environmental Protection Agency (USEPA) has begun to execute a new paradigm with the Endocrine Disruptor Screening Program (EDSP). The goal is to prioritize chemicals based on route of exposure and screen them for potential endocrine or thyroid activity. Screening is being conducted using 11 Tier 1 assays. One of those assays is the Amphibian Metamorphosis Assay. Although the OPPTS 890.1100 guideline is very specific, it is not a trivial matter to meet the acceptance and performance criteria listed in the assay. Even though this assay has been validated, there are still not enough baseline data to reliably set acceptance or rejection criteria. This presentation will evaluate control performance data from at least 7 assays conducted over the past 10 months and compare this performance against recommended acceptance and performance criteria presented in the OPPTS 890.1100 and OECD 231 guidelines. The endpoints evaluated will include: number of day to stage 51; Day 7 developmental stage, hind limb length, snout to vent length and weight; and Day 21 developmental stage, hind limb length, snout to vent length (SVL), weight and thyroid histopathology. Recommendations regarding acceptance and performance criteria for this guideline will be provided.

20.

The Evaluation of Control Performance in the Fish Short-Term Reproduction Assay Against the OPPTS 890.1350 Guideline

Duncan York, Lee E. Sayers, Ronald C. Biever
Smithers Viscient, Wareham, MA

The United States Environmental Protection Agency (USEPA) has begun to execute a new paradigm with the Endocrine Disruptor Screening Program (EDSP). The goal is to prioritize chemicals based on route of exposure and screen them for potential endocrine or thyroid activity. Screening is being conducted using 11 Tier 1 assays. One of those assays is the Fish Short-Term Reproduction Assay. Although the OPPTS 890.1350 guideline is very specific, it is not a trivial matter to meet the acceptance and performance criteria listed in the assay. Even though this assay has been validated, there are still not enough baseline data to reliably set acceptance or rejection criteria. This presentation will evaluate control performance data from at least 12 assays conducted over the past 10 months and compare this performance against recommended acceptance and performance criteria presented in the OPPTS 890.1350 and OECD 229 guidelines. The pre-exposure endpoints evaluated will include: fecundity expressed as eggs/female/day and number of spawns. The exposure endpoints evaluated will include: survival, fecundity expressed as eggs/female/day, percent fertility, male and female gonad histopathology, gonadal somatic index (GSI) and blood plasma vitellogenin, and male tubercle score. Recommendations regarding acceptance and performance criteria for this guideline will be provided.

21.

HPLC-ESI/MS/MS Method for the Determination of Endogenous Estradiol and Testosterone in Fish Plasma

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Smithers Viscient, Wareham, MA

Estradiol and testosterone are two key steroids of the endocrine system that can be measured as optional endpoints in the Fish Short-Term Reproduction Assay. The levels in plasma can be used as an indicator of disruption in endocrine system functions. An HPLC-ESI/MS/MS method was developed and validated for the simultaneous determination of both estradiol and testosterone in fish plasma. Whole blood was

collected manually from fathead minnows (Pimephales promelas). Plasma was generated by centrifugation. Analyses were performed on small volume plasma samples (generally ≤ 10 µL). Estradiol was derivitized with dansyl chloride. Testosterone was determined without modification. Deuterated internal standards for both analytes were utilized. The LOQ for the analysis is 0.3 ppb for estradiol and 0.15 ppb for testosterone. Endogenous levels for control fish generally agreed with published values. This method is being used in conjunction with Tier 1 Endocrine Disruptor Screening Program (EDSP) assays.

22.

Chlorpyrifos: Weight of Evidence Evaluation of Potential Interaction with the Estrogen, Androgen or Thyroid Pathways

Daland. R. Juberg[1], Sean C. Gehen[1], Vince J. Kramer[1], Haitian Lu[1], M. Sue Marty[2], Katie K. Coady[2]
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Chlorpyrifos was one of 67 chemicals selected for EPA's Endocrine Disruptor Screening Program (EDSP) based on widespread use and potential for human and environmental exposures. The purpose of the program is to screen pesticide chemicals for their potential to interact with the estrogen, androgen, or thyroid systems.

A battery of 11 EDSP assays covering the scope of the program was completed for chlorpyrifos based on the EDSP data call-in and in accordance with test guidelines developed for EDSP Tier 1 testing.

To assist with scientific interpretation of the EDSP screening results for chlorpyrifos and within the context of existing data from regulatory guideline studies and published literature, a weight-of-evidence (WoE) evaluation was completed. This WoE approach was based on the OECD conceptual framework for testing and assessment of potential endocrine-disrupting chemicals and consisted of a systematic evaluation of data, progressing from simple to complex across multiple levels of biological organization. The conclusion of the WoE evaluation is that chlorpyrifos demonstrates no potential to interact with the endocrine system, including estrogen, androgen, and thyroid pathways.

Based on the results of the Tier 1 testing for chlorpyrifos under the EDSP, and within the context of this WoE evaluation, there is no scientific justification for pursuing additional endocrine testing for chlorpyrifos.

23.

Comparison of *In Vivo* Toxicological Studies with Endocrine Disruptor Screening Program Tier 1 and High Throughput Assays: Atrazine Case Study

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Syngenta Crop Protection, LLC.

Atrazine, a widely used herbicide, was included in List 1 of the Tier 1 testing for the Endocrine Disruptor Screening Program (EDSP). However, most of the testing requirements for atrazine were waived since it has been previously tested in many assays that are similar or identical to the Tier 1 tests. In fact, atrazine has served as positive and negative controls for various EDSP Tier 1 and ToxCast™ assays. It is clear that atrazine is not estrogenic: negative in ER binding (rat uterine cytosol and Human ER transfected to yeast) and uterotrophic assays. Atrazine does not bind to androgen receptors for both recombinant and rat prostate cytosol. Atrazine was also negative in the Hershberger assay. Although atrazine administration resulted in delayed onset of puberty in both male and female rats at relatively high dose levels, there were no reproductive and developmental effects in other *in vivo* toxicological studies. Mode of action studies have demonstrated that high doses of atrazine accelerate the reproductive aging process in female Sprague-Dawley rats by indirectly blocking the pre-ovulatory luteinizing hormone surge and prolonging the estrus cycle, which is not relevant to humans. It has also been shown that young animals are less sensitive to these effects. The ToxCast™ and EDSP Tier 1 assays have not been consistently predictive of *in vivo* effects. In light of the total data including ToxCast™ and EDSP Tier 1 assays, the weight of evidence for atrazine demonstrates that further refinement is necessary for screening for potential endocrine disruptors.

24.

Retrospective Analysis of Syngenta Compounds – Predictive Value of ToxCast™ High-Throughput Assays for Endocrine Disruptor Screening Tier I Studies

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Syngenta Crop Protection, LLC

Eleven Syngenta compounds were included in the first list of the Tier I Endocrine Disruptor Screening Program (EDSP) administered by the United States Environmental Protection Agency (US EPA). Based on Other Scientifically Relevant Information (OSRI) assessments, the US EPA considered four of the eleven Syngenta compounds listed required the full battery of mammalian EDSP Tier I testing, ranging from the *in vitro* nuclear receptor binding assays to the *in vivo* male and female pubertal assays. Different methodologies used in ToxCast™ and *in vitro* EDSP Tier I assays prevents the use of ToxCast™ assays as an acceptable alternative to EDSP Tier I assays; however they are considered useful in prioritizing chemicals for EDSP Tier I testing. A retrospective analysis was conducted to assess the predictive value of the high-throughput ToxCast™ data on any endocrine disrupting potential of abamectin, chlorothalonil, metalaxyl and norflurazon. An additional assessment evaluated the impact of the nine mammalian EDSP Tier I assays had on advancing the understanding of any potential endocrine disrupting potential of these four compounds. The ToxCast™ and EDSP Tier I data did not identify abamectin, chlorothalonil, metalaxyl or norflurazon as potential endocrine disruptors, which is consistent with the current toxicology database for each compound. This retrospective analysis highlights a challenge to focus on: how can we close the knowledge gap between *in vivo* and *in vitro* endocrine disruptor testing and reduce the need for additional testing?

25.

2,4-Dichlorophenoxyacetic Acid (2,4-D): Updated Evaluation of Endocrine Modulating Potential

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We have performed a comprehensive weight-of-the-evidence review of both published and unpublished studies of 2,4-D. 2,4-D has a robust mammalian data base, including developmental, sub-chronic and chronic toxicity studies, as well as an F1-extended one generation reproductive toxicity study assessing potential endocrine toxicity, developmental neurotoxicity and immunotoxicity. These data show 2,4-D has a low potential for endocrine modulation (androgenic, anti-androgenic, estrogenic, anti-estrogenic or thyroid active) unless the threshold of renal clearance is exceeded, at doses not relevant to human exposure. In vitro mechanistic and screening assays also support a low likelihood of endocrine activity. The data base for endocrine evaluation of 2,4-D in non-mammalian species is limited; this has recently been supplemented by screening studies in Xenopus laevis tadpoles and fathead minnows; no endocrine-specific effects were observed. In vitro EDSP assays were negative. In mammals, doses of 2,4-D well above saturation of renal clearance are associated with thyroid findings. These effects are related to displacement of circulating thyroid hormone from transthyretin binding protein, thus enhancing metabolism and excretion of thyroid hormone. A detailed evaluation of thyroid function across life stages in rats, showed only adaptive changes in dams exposed to 2,4-D at a dose exceeding the renal clearance threshold; there were no adverse effects on offspring. Humans have higher levels of a high-affinity thyroid binding protein, i.e., thyroxine binding globulin, compared to the rat, which likely makes humans less sensitive to displacement of thyroid hormone by 2,4-D. The progression and rate of frog metamorphosis, which depends on thyroid function, was examined in 2,4-D-exposed tadpoles, and no treatment-related effects were observed up to the limit concentration. Thus, based on toxicity data in multiple species, there is low potential for 2,4-D to alter endocrine function at environmentally relevant concentrations.

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26.
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Seamless Product Development Solutions, ABC Laboratories

28.
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Battelle's EDSP Services: Tier 1 *In Vivo* Mechanistic Studies

Frizell, E.R., Fallacara, D., Kobs, C.L., Huwar, T.B., Brys, A.M., Essman-Wood, C., Skowronek, A.J., Singer, A.W., Gerken, D.K., and Sparrow, B.
Life Sciences Research, Battelle Memorial Institute, Columbus, OH

Proficiency studies were based on OECD validations for the Hershberger Bioassay [HBA] (Owens 2006) and the Uterotrophic Assay [UTA] (Kanno 2001), and conducted following EPA's current OPPTS 890 Guidelines.
HBA: Sprague-Dawley (SD) rats (9/group) were castrated on postnatal day (PND) 44. Five groups, used to test androgen agonism, received 0, 0.1, 0.2, 0.4, and 0.8 mg/kg/day testosterone propionate (TP) in corn oil, subcutaneously (s.c.). Two additional groups, used to test androgen antagonism, received 0.4 mg/kg/day TP s.c. Of these, the negative and positive groups also received corn oil alone and 3 mg/kg/day flutamide in corn oil, respectively, by oral gavage. In all cases, dosing occurred for 10 consecutive days starting on PND 55.
UTA: SD rats (9/group), were ovariectomized on PND 42. Five groups, used to test estrogen agonism, received 0, 0.1, 0.3, 1.0, and 3.0 µg/kg/day, 17-α ethynyl estradiol (EE) in corn oil, s.c., for 3 consecutive days, starting on PND 62.
Parameters evaluated were: viability, clinical signs, body weights, and food consumption.
For HBA the weight of five androgen sensitive organs [ventral prostate (VP), paired seminal vesicles (with fluid) plus coagulating glands (SV/CG), levator ani/bulbocavernosus muscle complex (LABC), glans penis (GP), and paired Cowper's glands (COW)] and serum levels of testosterone, lutenizing and follicle stimulating hormones, were measured. For UTA, the wet and blotted uterine weights were collected.
The results demonstrated Battelle's laboratory proficiency for the evaluation of pro- and anti-androgenic effects and pro-estrogenic effects required by the EDSP Tier 1 *in vivo* mechanistic studies.

Battelle's EDSP Services: Tier 1 Male and Female Pubertal Assays

Frizell, E.R., Fallacara, D., Toy, H., Brys, A.M., Essman-Wood, C., Vasconcelos, D., Skowronek, A.J., Singer, A.W., Gerken, D.K., and Sparrow, B.
Life Sciences Research Battelle Memorial Institute, Columbus, OH

Male and Female Pubertal Assays were conducted at Battelle following the EPA's Guidelines for Pubertal Development and Thyroid Function in Intact Peripubertal Male and Female Rats (OPPTS 890.1500 and 890.1450, respectively).
Environmental conditions, husbandry and procedures followed the Tier 1 Guidelines. Fifteen rats/sex/group were used. For the Female assay, the positive group was dosed with Tamoxifen (TAM) at 10 mg/kg/day from post-natal day (PND) 22 to 42. For the Male assay, the positive group was dosed with Dibutyl Phthalate (DBP) at 1000 mg/kg/day from PND 23 to 53. Male and female control rats received corn oil alone at the same dose volume as the positive groups (2.5 ml/Kg), daily, by oral gavage. Necropsies occurred on PND 23 and 22, for males and females, respectively.
Parameters evaluated were clinical observations, body weight, food consumption, age at achievement of preputial separation or vaginal opening, vaginal cytology, organ weights [pituitary, thyroid/parathyroid (post-fixation), adrenals, kidneys and liver; testes, epididymides, ventral and dorsolateral prostate, levator ani/bulbocavernosus muscle complex (LABC), seminal vesicles plus coagulating glands (with and without fluid); uterus with/without luminal fluid, and ovaries]. Histopathology included thyroid follicular height and colloid area scoring and evaluation of ovarian follicular development by PCNA staining. Terminal blood samples for serum chemistry parameters and hormones [T_4, TSH (and testosterone in males)] were collected within 2-3 minutes of anesthetic induction.

Linking High-Throughput In Vitro Screens with EDSP Assays: The Causal Relationship Challenge

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One step in complying with Test Orders under the US EPA's Endocrine Disruptor Screening Program was compilation of Other Scientifically Relevant Information (OSRI). The extent and quality of OSRI was evaluated to determine what requirements were met by existing data, and hence, which of the 11 Tier 1 Endocrine Screening Battery (ESB) assays were needed. Many of the List 1 chemicals under ESB Test Orders were analyzed in the EPA's ToxCast Program, hence ToxCast data were submitted as OSRI in lieu of conducting many similar ESB *in vitro* assays, and to help support waivers for some *in vivo* assays. ToxCast seeks to predict human toxicity by measuring the activity of chemicals in a suite of some 500 cellular, biochemical and molecular assays conducted in high-throughput mode. The concept presumes that these assays can identify a chemical's ability to activate so-called 'toxicity pathways.' In evaluating acceptability of OSRI, EPA rejected ToxCast results claiming that the reliability, responsiveness and relevance of ToxCast were undetermined and that ToxCast does not evaluate all known endocrine targets. In rejecting ToxCast data, EPA claimed its validation status was inferior to that of the individual ESB assays. However, even the predictive value of the *in vitro* ESB assays for *in vivo* endocrine activity is still unclear at this time. Although ToxCast data was rejected as OSRI for the ESB, ToxCast data was used to assess and deem acceptable the risks of oil dispersants used for the Deep Water Horizon spill. These decisions have significant implications for the use of screening assays in regulatory programs, yet inconsistencies in application confound what those implications might be. As both the ESB and ToxCast programs appear to rely on correlative analyses rather than dispositive evidence that assays individually or in combination measure precursor events or frank toxicity, the use of high-throughput assays, like ToxCast, to screen chemicals for further testing may be no less probative than some of the ESB battery. However, despite potential advantages offered by high-throughput methods to quickly screen for possible modes of action, it will be necessary to elucidate aspects of biological and physiological function as well as causal relationships between biochemical events and adverse effects before these data can offer improvements in the assessment of endocrine-mediated toxicity.

The Order of Things: How to Make the Most Out of the EPA's Tier 1 Endocrine Screening Battery

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EPA's Endocrine Disruptor Screening Program (EDSP) was initiated in 2009-2010 with the issuance of test orders requiring manufacturers and registrants of 58 pesticide active ingredients and 9 pesticide inert/high production volume chemicals to evaluate the potential for these chemicals to interact with the estrogen, androgen and thyroid hormone systems. The EPA Tier 1 endocrine screening battery (ESB) consists of 11 distinct assays comprising both in vitro and in vivo test systems. Much effort has gone into developing and standardizing these screens. However, there are still challenges in utilizing the results to identify a substance's potential to interact with the endocrine system of humans and wildlife as some of the ESB methods lack specificity for differentiating potential endocrine mediated responses from responses via other modes of action or via general toxicity. In addition, screening of compounds using the ESB is not a trivial undertaking as the ESB can take many years to complete and is estimated to cost $750,000 to $1,000,000 per chemical. To better understand the potential for substances to interact with endocrine pathways, a four-stage sequence is proposed to conducting the 11 assays comprising the EDSP Tier 1 ESB. The rationale for this sequence is to provide a better context in which to interpret the results of each individual assay in the ESB so that the battery as a whole can be interpreted more clearly and

consistently. Efficiency and clarity is essential since interpretation of the entire ESB is the determinant for proceeding to EDSP Tier 2, apical testing of reproductive and developmental life stages in several species to provide data on adverse effects and dose response for risk assessment.

Endocrine Disruption: Weight of the Evidence for Low-Dose Effects of TCDD on Sperm Counts

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Considerable debate is ongoing regarding the potential for endocrine-mediated, low dose effects. One of the examples that has been presented as evidence for low-dose effects is exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and the impact on sperm counts in male offspring. Although one epidemiological study has reported reduced sperm counts in males exposed to TCDD, the results of this study are limited by study design and methodology. We have conducted a weight-of-the-evidence review of the toxicological studies investigating the impact of maternal TCDD exposure on sperm counts in their offspring and the results have been highly variable. Epididymal sperm counts have been reported to be reduced at doses as low as 64 ng TCDD/kg, but other studies have failed to show effects at doses of 800 ng TCDD/kg or higher. Several other animal studies have not reported effects on sperm counts at doses between 10 and 50 ng TCDD/kg, suggesting a potential threshold. While sperm effects have been reported in experimental animal studies at 64 ng TCDD/kg, this dose should not be considered a low dose based on current intakes. Thus, discussions regarding the potential endocrine-disruptive effects of TCDD on sperm counts are misleading; the effects do not occur at the low doses experienced by the general American public.