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PEOPLE FOR THE ETHICAL TREATMENT OF ANIMALS

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Re: **Endocrine Disruptor Screening Program Tier 1 Screening Order Issuing Announcement (74 FR 54422); EPA–HQ–OPP–2009–0634**

The accompanying comments are being submitted on behalf of the more than two million members and supporters of People for the Ethical Treatment of Animals who are concerned about promoting reliable and relevant toxicity testing strategies that protect human health and the environment while reducing, and ultimately eliminating, the use of animals. Our comments are submitted in response to issuance of Tier 1 Screening Orders for the Environmental Protection Agency's (EPA) Endocrine Disruptor Screening Program (EDSP) for permethrin issued on November 19, 2009, under the request for existing data and "other scientifically relevant information" (OSRI) in which "persons other than recipients" have 90 days to respond.

Introduction

EPA has initiated the EDSP Tier 1 screening for the first group of 67 chemicals by issuing test orders from October 29, 2009, through February 26, 2010. The 67 Phase I chemicals consist of 58 pesticide active and nine High Production Volume (HPV) chemicals used as pesticide inert ingredients (also known as "pesticide inerts"). These chemicals were chosen for testing based on exposure potential considering four exposure pathways for each type of chemical. The four exposure pathways identified for pesticide active ingredients are: food, drinking water, residential use, and occupational exposure. The four exposure pathways identified for HPV/pesticide inert chemicals are: human biological monitoring, ecological biomonitoring, drinking water, and indoor air.[1]

These chemicals are to be tested in five *in vitro* and six *in vivo* assays (**Table 1**). The stated purpose of the Tier 1 battery is to "identify substances that have the potential to interact with the EAT [estrogen/androgen/thyroid] hormonal systems…".[2] The EPA has stated that it intends to use a weight-of-evidence approach to evaluate the results of the Tier 1 studies,[3] and based on this assessment, EPA will determine which, if any, of the Tier 2 tests are necessary. The putative Tier 2 battery consists of developmental and reproductive toxicity tests in several vertebrate species and is designed to identify and establish dose-response relationships for any adverse endocrine-related effects.

These comments address the test orders for endosulfan, a widely used organochlorine insecticide that has been extensively tested as part of registration. This testing involves dozens of toxicity tests in vertebrate animals, including reproductive and chronic/lifecycle studies in rodents, fish and birds, as well as metabolism and pharmacokinetics studies.[4] These tests kill thousands of

animals and include many of the same endpoints addressed in the presumptive EDSP Tier 2 tests (**Table 2**).

These comments are a continuation in a series of comments on individual Phase I chemicals from PETA and the Physicians Committee for Responsible Medicine.

References

[1] 74 FR 17579. April 15, 2009; EPA Final List of Initial Pesticide Active Ingredients and Pesticide Inert
Ingredients to be Screened Under the Federal Food, Drug, and Cosmetic Act.

[2] 74 FR54415, October21, 2009. Endocrine Disruptor Screening Program (EDSP); Announcing the
Availability of the Tier 1 Screening Battery and Related Test Guidelines; Notice.

[3] Response to Comments on the Public Review Draft of the Information Collection Request (ECR) entitled "Tier 1 Screening of Certain Chemicals Under the Endocrine Disruptor Screening Program (EDSP)", contained in Docket ID no. EPA-HQ-OPPT-2007-1081, page 16.

[4] 72 FR 60934, October 26, 2007: EPA 40 CFR Parts 9 and 158: Pesticides; Data Requirements for Conventional Chemicals.

Table 1: EDSP Tier 1 Assays

	Species	Mechanism addressed	Endpoints	suggested equivalent information
in vitro				
ER TA: OPPTS 890.1300 OECD TG 455	endogenous human ERα	Estrogen agonists	ERa-dependent transcriptional activation	effect ovary/uterus size, histology, male/female fertility
ER binding OPPTS 890.1250	Rat uterine cytosol	Estrogen agonists, antagonists	ER binding	effect ovary/uterus size, histology, male/female fertility
AR binding: OPPTS 890.1150	rat prostate cytosol	Androgen agonists, antagonists	AR binding	effect on testes size, histology, male/female fertility
Steroidogenesis - H295R OPPTS 890.1550	human	Steroid synthesis (estrogen and testosterone)	testosterone, estrogen hormone levels	effect on estrogen/testosterone levels, sex organs, male/female fertility
Aromatase OPPTS 890.1200	human	Aromatase inhibition, the enzyme responsible for the conversion of androgens to estrogens	$^3H_20$ released during the conversion of androstenedione to estrone	effect on estrogen/testosterone levels, sex organs, male/female fertility
In vivo:				
Uterotrophic OPPTS 890.1600 OECD TG 440	rat, mouse immature: PND 18 - 21 ovarectimized: 6 - 8 weeks	Estrogen agonists, antagonists (in GD, not well developed)	body weight, uterine weight, optional: histolopathology of vagina	evidence of estrogenic activity, uterine or vaginal weight changes, uterine or vaginal histology, effects on fertility reproduction
Hershberger OPPTS 890.1400 OECD TG 441	rat, mouse	Androgen agonists, antagonists, and 5α-reductase inhibitors	ventral prostate (VP), seminal vesicle (SV), levator ani-bulbocavernosus (LABC) muscle, paired Cowper's glands (COW) and the glans penis (GP)	evidence of androgenic activity, male sex organ weights or histology, effects on fertility reproduction

Pubertal female OPPTS 890.1450	rat	Anti-thyroid, estrogenic or anti-estrogenic (including alterations in receptor binding or steroidogenesis), luteinizing hormone, follicle stimulating hormone, prolactin or growth hormone levels or via alterations in hypothalamic function	Growth (daily body weight), Age and body weight at vaginal opening, Organ weights: Uterus, Ovaries, Thyroid, Liver, Kidneys, Pituitary, Adrenals. Histology: Uterus, Ovary, Thyroid, Kidney. Hormones: Serum thyroxine (T4), Serum thyroid stimulating hormone (TSH). Estrous cyclicity: Age at first estrus, length of cycle, percent of animals cycling. Standard blood panel, including creatinine and blood urea nitrogen.	evidence of estrogenic or thyroid activity, uterine or vaginal weight changes, uterine or vaginal histology, effects on fertility reproduction
Pubertal male OPPTS 890.1500	rat	Anti-thyroid, androgenic, or anti-androgenic [androgen receptor (AR) or steroid-enzyme-mediated], alterations in gonadotropins, prolactin, or hypothalamic function	Growth (daily body weight), Age and body weight at preputial separation, Organ weights: Seminal vesicle plus coagulating gs, Ventral prostate, Dorsolateral prostate, Levator ani/bulbocavernosus muscle complex, Epididymides, Testes, Thyroid, Liver, Kidneys, Pituitary, Adrenals. Histology: Epididymis, Testis, Thyroid, Kidney. Hormones: Serum testosterone, Serum thyroxine (T4), Serum thyroid stimulating hormone (TSH). Standard blood panel, including creatinine and blood urea nitrogen.	evidence of androgenic or thyroid activity, male sex organ weights or histology, effects on fertility reproduction
Amphibian metamorphosis OPPTS 890.1100	*Xenopus laevis*	hypothalamic-pituitary-thyroid (HPT) axis, Androgen agonists, antagonists, testosterone synthesis	Day 5: developmental assessment: hind limb and body length, body weight, developmental stage. Day 21 (termination): Developmental stage, SVL, hind limb length and wet body weight, thyroid gland histology.	evidence of androgenic or thyroid activity, male sex organ weights or histology, effects on fertility reproduction
Fish short-term reproductive screen OPPTS 890.1350 OECD 229	fathead minnow	hypothalamus-pituitary-gonadal (HPG) axis	survival, reproductive behavior, secondary sexual characteristics (number and size of nuptial tubercles), gonadal histopathology, gonado-somatic index, plasma concentrations of vitellogenin, 17β-estradiol and testosterone, fecundity (# eggs/female), fertility (%embryos/eggs)	evidence of estrogenic/androgenic activity, effects on fertility of reproduction

Table 2: Pesticide Data requirements related to EDC

Toxicological data requirements			**Use**	
OPPT guideline		**Relevant endpoints**	**food**	**non-food**
870.4100	Chronic oral: rodent	12 months exposure: gross necropsy plus histopathology of liver, kidneys, adrenals, testes, epididymides, ovaries, uterus, thyroid (with parathyroid), spleen, brain	R	CR
870.6200	90-day neurotoxicity	FOB: autonomic function (lacrimation, salvation, etc), convulsions, tremors, abnormal motor movements, reactivity to general stimuli (no reaction to hyperreactivity), general level of activity (unresponsive to hyperactive), posture and gait abnormalities, forelimb and hindlimb grip strength, foot splay, sensorimotor responses, body weight, neuropathology.	R	R
870.4200	Carcinogenicity	24 month exposure: clinical observations, blood smears, gross necropsy, possible histopathology of salivary glands, esophagus, stomach, intestine, liver, pancreas, gallbladder, brain, pituitary, peripheral nerve , spinal cord , eyes, adrenals, parathyroid, thyroid, trachea, lungs, pharynx, larynx, nose. aorta, heart, bone marrow, lymph nodes, spleen, kidneys, urinary bladder, prostate, testes, epididymides, seminal vesicle(s), uterus, ovaries, female mammary gland, all gross lesions and masses, skin.	R	CR
870.3700	Prenatal developmental toxicity, rat and rabbit	Exposure througout gestation: fetal deaths, resoption, sex and weight of each fetus, skeletal and soft-tissue abnormalities of fetuses	R	R
870.3800	Reproduction and fertility	Standard 2-gen: integrity and performance of the male and female reproductive systems, including gonadal function, the estrous cycle, mating behavior, conception, gestation, parturition, lactation, and weaning, and on the growth and development of the offspring. **P animals:** Cycling in females, sperm count, morphology, motility in males. Organ weights: uterus , ovaries, testes, epididymides, seminal vesicles, prostate, brain, pituitary, liver, kidneys, adrenal glands, spleen. Hisotpathology of vagina, uterus with oviducts, cervix, and ovaries, testis, epididymis, seminal vesicles, prostate, coagulating gland, pituitary and adrenal glands. **F1:** weight and gross abnormalities throughout developement, age of vaginal opening and preputial separation, anogenital distance, same organ weights as P, same histopath as P. **F2 weanlings:** histopathological examination of treatment-related abnormalities.	R	R
870.6300*	Developmental neurotoxicity	Perinatal exposure. Pup weight during growth, gross developmental abnormalities, motor activity, learning and memory, neuropathology (brain)	R	CR
870.7800*	Immunotoxicity	Functional tests: either antibody plaque-forming cell (PFC) assay or ELISA-based antibody reaction, NK cell activity. Cell counts of splenic or peripheral blood total B cells, total T cells, and T cell subpopulations.	R	R

Terrestrial and aquatic non-target organism data requirements			Use				
			terrestrial	aquatic	forestry	residential	Greenhouse/ indoor
850.2300	Avian reproduction	Eggs laid, percent fertilized, eggs not cracked, shell thickness, hatching, chick survival	R	R	R	R	NR
850.1400 (OECD TG 210)	Fish early life stage (freshwater)	Exposure of eggs until hatching: cumulative mortality, numbers of healthy fish at end of test, time to start of hatching and end of hatching, numbers of larvae hatching each day, length and weight of surviving animals, numbers of deformed larvae, numbers of fish exhibiting abnormal behavior.	R	R	R	R	NR
850.1500	Fish life cycle	Locomotion, behavioral, physiological, and pathological effects, spawning, egg numbers, fertility, and fecundity.	CR	CR	CR	CR	NR

*new in 2007

Endosulfan, CAS 115-29-7
Test Order Numbers: EDSP-079401-102 through 104
Test Order Date: December 3, 2009

Introduction: Endosulfan (CAS 115-29-7), a dioxathiepin (broadly classified as an organochlorine), is a broad spectrum contact insecticide that is used on a wide variety of vegetables, fruits, cereals, and cotton, as well as ornamental shrubs, trees, vines, and ornamental herbaceous plants in commercial agricultural settings. Endosulfan was first registered in 1954 to control a broad spectrum of agricultural insect and mite pests on various crops. Use data from 1987 to 1997 indicate an average domestic use of approximately 1.38 million pounds of active ingredient per year. Endosulfan generally has been shown to have high acute oral and inhalation toxicity as well as slight dermal toxicity. It is an irritant to the eyes and is not a dermal sensitizer. Endosulfan does not appear to have mutagenic or carcinogenic effects. Endosulfan primarily affects the nervous system and neurotoxicity is seen in insects and mammals, including humans. *Developmental neurotoxicity* and chronic/carcinogenic toxicity studies found that endosulfan causes *neurotoxic effects*, which are believed to result from over-stimulation of the central nervous system due to antagonistic activity affecting the *GABA-gated chloride channel*. The no observed effect levels (LOELs) for neurotoxicity have been reported to be between 0.6 and 1.5 mg/kg/day depending on endpoint and route of exposure (summarized by Silva and Beauvais 2010).

It is interesting to note that, several times during the regulatory history of endosulfan, public and private groups have tried to have the pesticide banned. In 2000 US EPA prohibited household use of endosulfan. In 2002 the US Fish and Wildlife Service recommended to US EPA that all uses of endosulfan be prohibited. In February of 2008, environmental, consumer, and farm labor groups including the Natural Resources Defense Council, Organic Consumers Association, and the United Farm Workers call on the U.S. EPA to ban endosulfan. In May of 2008, coalitions of scientists, environmental groups, and arctic tribes ask the EPA to cancel endosulfan, and in July 2008 a coalition of environmental and workers groups file a lawsuit against the EPA challenging its 2002 decision to not ban it.

Endosulfan continues to be regulated by US EPA as a Category I (highly acutely toxic) pesticide based on an LD50 of 30mg/kg for rats, an LD50 of 9.8-10.2mg/kg for mice (RED 2002).

Assessment of estrogenic activity: To test the hypothesis that a combination of potentially weak estrogenic agonists may have an amplified effedt when combined, Wistar rats were treated with a combination of endosulfan and deltamethrin in a *2 generation reproductive toxicity* test. Dams received 1.5mg/kg, 2.0mg/kg, or 3.0mg/kg endosulfan, plus 2.0mg/kg, 3.0mg/kg, or 4.0mg/kg of deltamethrin daily from gestational day 6 to day 21 of lactation. Female offspring were divided for a *uterotrophic assay* at 3 days or at puberty. The dams did not show any signs of maternal toxicity at any dose level. Gestation length, litter size, sex ratio, bodyweight of offspring, and the number of viable pups were also unaffected. Relative uterine weight of immature rats that received endosulfan during gestation and lactation were not affected. No adverse *pubertal* effects were observed in the sexual development of females exposed *in utero* and during lactation, nor in females exposed at puberty. No statistically significant changes were observed at vaginal opening or at first estrus cycle, cycle length, or time to reach fourth estrus as compared to vehicle control group. The administration of endosulfan did not affect sexual organ weights of females treated during gestation and lactation (Presibella 2005). An increase of uterine

weight is generally considered to be the best indicator of estrogenicity and in this study, *no effect on uterine weights* from immature rat offspring exposed from the fetal phase.
In another study using female Swiss albino mice, endosulfan was administered daily at 1.5, 3, 6, and 9mg/kg for 15 days. Mice were either hemicastrated (hemi-ovarectimized) or sham-operated observed for pesticide effects on ovarian compensatory hypertrophy. No significant change was observed in body weight, nor in the weights of uterus, kidney, adrenal, liver, thymus, and *thyroid* of endosulfan treated mice as compared to olive oil treated control group. LD50 is reported at 9.8-10.2mg/kg (Hiremath 2002).
In an *in vitro* study using a yeast reporter gene assay, the combination of 11 possible xenoestrogens (including endosulfan) affected the actions of 17β-estradiol (Rajapakse 2002). This study emulates simultaneous exposure to numerous chemicals and the additive effect they may have in humans. However, due to the high potency of 17β-estradiol, researchers concluded that the observed effects of the endosulfan-estradiol (mixture) effects were possibly due almost entirely to the action of the steroid hormone and not to the pesticide.
Male reproductive effects: In a *reproductive toxicity* test, female Wistar rats were fed endosulfan at 0.5mg/kg or 1.5mg/kg 21 days prior to mating, during the mating, pregnancy, and lactation. Male offspring were evaluated for sexual development, sex organ weights, sperm production, sperm morphology, and testosterone levels. Maternal weight reduction was not observed during the treatment. Litter size, number of viable pups, and body weights of pups at birth and at weaning were unaffected. The age of testis descent did not differ among groups. The testis, prostate, and seminal vesicle absolute and relative weights were unaffected at either dose tested. *In utero* exposure to endosulfan did not affect sperm count, sperm production, sperm morphology, and serum testosterone of the adult male offspring (**Table 1**, Dalsenter 2003).

Endosulfan had no effect on male reproductive development and sexual maturity.

Estrogenicity, Androgenicity, and Aromatase Activity *in vitro*: Estrogenicity, androgenicity, and aromatase activity of endosulfan was assessed using two cell lines (Chinese Hamster Ovary or "CHO" and human breast cancer or "MCF-7"), including both cytotoxicity and hormonal effects. In CHO cells, endosulfan behaved as a very weak anti-androgen at 20μM (activity was reduced to 68% of the control) with no agonist activity. Cell proliferation of MCF-7 cells was slightly higher when cells were treated with endosulfan. LOEC was reported at 1μM for MCF-7 cells in both cell proliferation and ER transactivation assay. Endosulfan at 50μM had a slightly inhibitory effect on aromatase, reducing activity to 87% of the control when using human placental microsomes. Cytotoxicity was seen in CHO cells at >100μM and in MCF-7 cells at >25μM (Andersen 2002).
In the studies above, endosulfan was shown to have no adverse effect on uterine weight, pubertal development, and estrous cycling. *In vitro*, endosulfan (even in combination with 10 other chemicals suspected of xenoestrogenic activity) did not have a negative impact on 17β-estradiol that could be attributed solely to the chemicals tested.

Fish and Amphibians: In a *developmental toxicity* test, larvae of *Rana pipens* were exposed daily for 7 weeks to one of three levels (0.2, 1, and 5μg/L)of endosulfan during growth and development. Growth rates of leopard frog tadpoles, quantified as tadpole length, were not affected at any dose level (Shenoy 2009).

In a *developmental toxicity* test, zebrafish larvae were exposed to two degradation products of endosulfan (endosulfan I between 0.01 and 1000μg/L and endosulfan sulfate between 1 and 10,000μg/L) did not cause any symptoms of overt toxicity at the highest levels tested. At ≥100μg/L (endosulfan I) a curved body axis and wavy notochord were seen in zebrafish larvae. Larvae also exhibited a sensitivity to touch at levels of ≥1μg/L which is likely due to neurotoxic effects commonly seen in other animals. The LOAEC for neurotoxic effects was established at 1μg/L for endosulfan I and 10μg/L for endosulfan sulfate (Stanley 2009).

Growth and development of fish and amphibians were either not affected, or affected at levels lower than those causing neurotoxicity.

Summary and Recommendations: The California EPA Department of Pesticide Regulation recently published a review article that has more than 130 research references for toxicity studies using endosulfan dating back to 1974 (Silva 2009). There is no conclusive evidence of endocrine disrupting activity *in vitro* or *in vivo* at levels lower than those that cause neurotoxicity. There is, however, clear evidence that endosulfan causes neurotoxicity in humans and rodents (LOEL is 0.61mg/kg) and is acutely toxic at low concentrations (LD50 as low as 9mg/kg). ***No additional studies are needed to evaluate endocrine disruption activity because developmental and reproductive toxicity data is readily available and endosulfan is already regulated based on its primary toxicity, neurotoxicity.***

References:

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Table 1
Reproduction Effects in Young Males Versus Adults (M/F) After Endosulfan Exposure

Author	Strain of animal source[a]	Treatment duration	No./dose	LOEL/NOEL (mg/kg/d)[c]
Effects in rats				
Sinha et al. (1995)	Druckrey[b] (ITRC India)	90-d-old M+70d treatment	5 M	LOEL = 2.5 (LDT) M Repro
Sinha et al. (1997)	Druckrey[b] (ITRC India)	3-week-old M pups; 90d, 5d/wk	5 M	LOEL = 2.5 (LDT) M Repro
Sinha et al. (2001a)	Druckrey[b] (ITRC India)	GD12-birth, x-fostered to PND 100	3 dams	LOEL = 1.0 (LDT) M Repro
Dikshith et al. (1984)	Wistar[b] (ITRC, India)	Adult M gavage 30d prior to mating	5 M/10F	Repro NOEL >5.0 M; 1.5 F NOEL = 2.5 M; 0.75 F
Dalsenter et al. (1999)	Wistar[b] (FURGS, Brazil)	Dams GD15–LD22; obs PND 65, 140; mate	8 Dams F1	Repro M Pup LOEL d65 = 1.5
		F1 M to virgin F PND 120; F2 obs PND 21[d]	8 litters F2	Repro M NOEL d120 & 140 >1.5
				Dam NOEL = 1.5
Dalsenter et al. (2003)	Wistar[b] (FURGS, Brazil)	21-d premating-weaning Observed PND140	8 M	Repro Male NOEL >1.5
				Dam & M Pup NOEL >1.5
Zhu et al. (2000)	Wistar[b] (Chinese stock, China)	Gestation-PND28	10 M	Repro Dam & M Pup NOEL >2.5
				Dam NOEL = 1.0[f]
Gilmore et al. (2006)[g,h]	Wistar Crl:WI(Han)[b] (CR, NC)	GD6-LD21; observations PND 21 (sperm)	23 litters;	Repro Dam & M Pup NOEL >29.8
			4 M/litter	Dam & Pup LOEL < 3.74 (LDT)[e]
Edwards et al. (1984)[g,h]	Crl:COBS(CD)BR[b] (CR, UK)	2 Generation; 2 litters/generation	28–30 M/F	Repro Dam & Pup NOEL >5.9
				Adult Pup NOEL = 1.2
Fung (1980a,b)[g,h]	CD SD[b] (CR, MI)	GD6–19	28 dams	Developmental Fetal NOEL = 2.0
				Dam NOEL = 2.0
Effects observed in rabbits				
Nye (1981)[g,h]	NZ W (HU, WI) Rabbit	GD6–28	26 dams	Developmental Fetal NOEL >1.8
				Dam NOEL = 0.7

LD = lactation day; GD = gestation day; PND = postnatal day; M = male; F = female; obs = observations; LDT = lowest dose tested; HDT = highest dose tested; NZ W=New Zealand White rabbits; Repro=reproductive Grey, *italics* = Studies where clinical observations for neurotoxicity were not reported. Grey, bold = Studies where NOELs for reproduction or developmental effects exceed the HDT and/or systemic NOEL.

[a]Abbreviations for animals and their animal sources: ITRC = Industrial Toxicology Research Centre, Lucknow, India; Federal University of Rio Grande do Sul, Brazil; HU = Hoppers Unlimited; NZ W = New Zealand White rabbits; CR = Charles River Laboratories: WI = Wisconsin, MI = Michigan; UK = United Kingdom; NC = North Carolina.

[b]Rat strains.

[c]Reproductive effects may include: sperm count, sperm production, sperm motility, sperm morphology, testes weights, epididymal weights, fertility, mating, litter, or offspring effects.

[d]F1 males were examined PND 65 and 140 (8 dams treated/dose); F1 PND 120 15 males/dose mated to virgin females. Pregnancy outcome and F2 pup observed to PND 21.

[e]The short-term decrease in body weight in pups at the LDT only was likely due to decreased palatability of diet as pups transitioned from nursing to treated diet prior to weaning. Pups at the higher doses did not recover from the decreased body weight effect.

[f]Four out of ten dams died and there were clinical signs of neurotoxicity in dams at 2.5 mg/kg/d.

[g]Studies performed according to Good Laboratory Standards in Brazil, the United Kingdom, or the United States.

[h]FIFRA Guideline studies.