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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 the second 12 chemicals, issued on November, 5, 2009, under the request for existing data and "other scientifically relevant information" (OSRI) in which "persons other than recipients" have 90 days to respond. We are responding for only two chemicals, chlorpyriphos and malathion. These comments extend those submitted by PETA and Physician's Committee for Responsible Medicine on February 5, 2010.

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 the first seven chemicals: atrazine, 2, 4-D, benfluralin, dimethyl tetrachloroterephthalate (DCPA), fenbutatin oxide, norflurazon, and propargite.[4,5] All seven of these chemicals are herbicide or insecticide active ingredients, and are therefore subject to extensive testing for pesticide 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.[6] These tests kill thousands of animals and include many of the same endpoints addressed in the presumptive EDSP Tier 2 tests (**Table 2**).

In its letter to EPA approving the Information Collection Request, OMB instructed EPA to "promote and encourage test order recipients to submit Other Scientifically Relevant Information (OSRI) *in lieu* of performing all or some of the Tier I assays, and EPA should accept OSRI as sufficient to satisfy the test orders to the greatest extent possible." In the interest of increasing the efficiency of the EDSP, the comments before provide existing data and OSRI in support of these OMB instructions to EPA, focusing on animal testing and vertebrate testing in particular. There is one section for each of the seven chemicals; references follow each section.

In all cases, the equivalent of Tier 2 (reproductive toxicity in one or more generations) information is available for rodents and in some cases also for fish and birds. There are two primary reasons for carrying out Tier 1 testing: 1) to discern mechanistic information about a chemical (i.e. does it function by interacting with the E, A or T hormone system) and 2) to evaluate what, if any, Tier 2 testing is warranted. Thus, if Tier 2 data already exist for a chemical, there is very little rationale for performing Tier 1 testing.

EPA has not articulated how endocrine disrupting chemicals would be regulated based on mechanism of action. Even though there is no precedence for such regulation to date, future regulation may benefit from mechanism of action information; in fact such information is critical for reduced dependency on whole animal testing and for improving the accuracy of hazard and risk determination as outlined in the 2007 NRC report: Toxicity Testing for the 21st Century: a Vision and a Strategy.[7] Rather than using whole animal tests such as the uterotrophic or Hershberger simply because they are available, mechanistic information can be obtained through non-animal means, in binding, transcriptional activation, or other cell-based systems, some of which are in use by the EPA's ToxCast™ program. A more efficient structure for the EDSP would be to start with a series of mechanistic *in vitro* assays to determine which, if any, of the endocrine pathways a chemical interacts with, and target any further testing accordingly.

The EPA's ToxCast™ program profiled 56 of the 73 EDSP Phase I chemicals, including atrazine, 2,4-D, benfluralin, norflurazon and propargite, in 14 assays directly related to endocrine activity (including estrogen, androgen, thyroid, and aromatase), and in an expanded set of 78 high throughput assays, including nuclear receptor and CYP450-related assays.[8] The advantage of the structure of the ToxCast™ program's database is that connections can rapidly be made between *in vitro* assay results and existing mammalian and ecotoxicity data, which greatly facilitates identification and interpretation of mechanism of action information.

Preliminary results from Phase I of the entire ToxCast™ program are promising.[9] Linkages between high-throughput *in vitro* results and *in vivo* endpoints can be made, and potency

rankings for groups or classes of chemicals are also being explored. Intriguingly, high "activity" across a large number of molecular pathways correlates inversely with lowest observed effect level (LOEL) in mammalian studies.

Rather than a default application of the full battery of Tier 1 assays to data-rich chemicals such as pesticides, a more efficient and potentially more useful approach would be to evaluate the existing relevant data, reproductive and developmental information in particular, in combination with information from a series of *in vitro* mechanistic assays such as those included in the Tier 1 and in ToxCast™, to determine what, if any, further testing is warranted.

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] 74 FR 54422, October 21, 2009; Endocrine Disruptor Screening Program; Tier 1 Screening Order Issuing Announcement, Order Issuance Schedule.

[5] EPA Endocrine Disruptor Screening Program, status of EDSP Orders/DCIs (http://www.epa.gov/endo/pubs/edsp_orders_status_012810.pdf; accessed 3 February 2010)

[6] 72 FR 60934, October 26, 2007: EPA 40 CFR Parts 9 and 158: Pesticides; Data Requirements for Conventional Chemicals.

[7] NRC (Committee on Toxicity Testing and Assessment of Environmental Agents, National Research Council). 2007. Toxicity Testing in the 21st Century: A Vision and a Strategy. National Academies Press, Washington, DC. Available at: http://www.nap.edu/catalog.php?record_id=11970. Accessed 25 January 2009.

[8] Kavlock et al. (2009) Biological Profiling of Endocrine Related Effects of Chemicals in ToxCast™. Poster presentation available at http://www.epa.gov/NCCT/toxcast/files/summit/40P%20Kavlock%20TDAS.pdf. Accessed February 4, 2010.

[9] Judson et al. (2009) "The Toxicity Data Landscape for Environmental Chemicals" Environmental Health Perspectives, Volume 117, Number 5, May 2009 (http://ehp.niehs.nih.gov/members/2008/0800168/0800168.pdf, accessed 5 February 2010).

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

Malathion, CAS number 121-75-5
Test order numbers EDSP-057701-43 through 49
Test order date: November 5, 2009

I. Introduction

Malathion, a broad-spectrum organophosphate (OP) insecticide, has been commercially available since its first registration in 1956 and is among the most widely used insecticides in agricultural and residential applications (Pluth J. et al. 1998). As a class, OPs exert their primary toxic effect in mammals by inactivating acetylcholinesterase (AChE), leading to acetylcholine (Ach) accumulation in the autonomous and somatic nervous system and eventual paralysis due to overstimulation of muscarinic and nicotinic receptors.

Malathion has been routinely applied as part of regional pest eradication and public health programs targeting the boll weevil, medfly and mosquito. Likely contributors to malathion's popularity include its perceived low acute toxicity in mammals, short soil persistence, and relatively quick degradation (Kaur S. et al. 2005). Nevertheless, malathion is an alkylating agent and therefore can produce genotoxic damage (Giri S. et al. 2002), and it has also been reported to produce *Hprt* mutations preferentially at G:C base pairs in human T lymphocytes (Pluth J. et al. 1998). Like other OPs, malathion is biologically activated by cytochrome P450. Malaoxon, the bioactivated metabolite, is significantly more toxic than its parent compound via the same AChE inhibitory mechanism (Galloway T. and Handy R. 2003; Costa L. et al. 2005). At the time of malathion's 2006 EPA Reregistration Eligibility Decision (RED), annual use approached 15 million pounds. Monitoring data from the USGS National Water Quality Assessment program (NAWQA) between 1992 and 2001 found malathion at a maximum concentration of 0.648 parts per billion (ppb) in samples from urban streams. Taking into account that the doses indicated for general agriculture are between 0.175 and 6.25 pounds of malathion active ingredient per acre, the estimated environmental concentration in water possibly ranges between 291 and 2.94 ppb that represent exposures far below the NOAEL and LOAELs established in reproductive and developmental toxicity studies (EPA 2009). A slight but significant decrease in the number of embryonic implantations using Sprague-Dawley rats, for instance, required malathion exposures on the order of 500 mg/kg/day during gestation (Prabhakaran S. et al. 1993).

Malathion has been shown to affect reproductive capacity in both sexes of several species and is considered a neurotoxicant. Early developmental exposure to neurotoxicants may perturb neural development and subsequent neurobehavior (Karczmar A. et al. 1970; Yanai J. et al. 2004), and cholinesterases may be involved in gamete function (Falugi C. et al. 1991) with a possible link to endocrine activity in several species (Angelini C. et al. 2004). Studies have reported neurotoxic effects of malathion exposure in both humans (Komori T. et al. 1991; Vidair C. 2004) and animals (Abdel-Rahman A. et al. 2004; Brocardo P. et al. 2005), and, as a result, malathion's reproductive toxicity has been examined in depth.

A. Reregistration Eligibility Decision and follow-up, 2006—2009

In May of 2009, EPA issued a revision to the 2006 RED for malathion following the receipt of results of a comparative cholinesterase study of malathion and malaoxon as well as an aerobic aquatic metabolism study of malathion alone. *To date, no fewer than* ***nine in vivo mammalian reproductive and developmental studies have been conducted whose results should be carefully reviewed to satisfy applicable DCI data requirements.***

In the available studies, as summarized in the 2009 revised RED, "there was no estrogen or androgen mediated toxicity", although thyroid effects had been noted in a 1979 NCI study using Fischer-344 (F344) rats. Convened in August of 2000 to assess classification of malathion's carcinogenic potential, the FIFRA Scientific Advisory Panel (SAP) evaluated existing chronic toxicity, carcinogenicity, subchronic toxicity and mutagenicity studies, concluding that "***observed thyroid tumors in male F344 rats and interstitial cell testicular tumors in male F344 rats were not significant to regulation because the tumor increase occurred only under conditions of toxicity that are not relevant to humans… there is neither a positive or biologically significant tumor response for any organ site after discounting dose groups in which there was marked toxicity. The tumor responses noted in these studies were unequivocally a result of excessive toxicity and have no relevance to any possible exposure scenario that could be encountered by human***" (FIFRA Scientific Advisory Panel 2000). Following a lack of observed treatment-related germ tissue or thyroid neoplasticity in a more recent study using F344 rats orally administered 1 to 141 mg/kg/day for 103 weeks (Daly 1996b), EPA commented that "thyroid effects were observed in the combined chronic/carcinogenicity study in rats, which included an increase in parathyroid hyperplasias in male and female rats, and a significant trend in thyroid follicular cell adenomas and/or carcinomas and thyroid c-cell carcinomas (all in males). However, the FIFRA Scientific Advisory Panel (SAP) did not consider the thyroid effects of concern or necessarily related to malathion exposure" (EPA 2009).

II. Existing toxicological and experimental data related to endocrine function

The effect of malathion on vertebrate development and reproduction has been studied in several species. Exploratory *in vitro* and *in vivo* experiments have demonstrated that malathion affects sperm count and motility in mammals (Akbarsha M. et al. 2000; Giri S. et al. 2002; Bustos-Obregon E. and Gonzalez-Hormazabal P. 2003). Post-exposure morphological changes in mouse testes, germ cell degeneration in birds, and germ cell mutagenicity in mice have been noted in the literature, although the mechanisms by which these effects occur are disputed (Contreras H. and Boustos-Obregon E. 1999; Maitra S. and Sarkar R. 1996). Indeed, evidence suggests that malathion's reproductive and/or endocrine-related effects may be indirectly mediated or potentiated by cell- and tissue-specific mechanisms, for example protein transport or DNA damage (see below)(Aluigi et al. 2005).

Changes in plasma hormone concentrations have been described following malathion exposure in *Heteropneustes fossilis* (Dutta H. et al. 1994), but *in vitro* reporter gene assays of estrogen and androgen receptors using Chinese haster ovary cells found no significant estrogenic or androgenic potential for malathion (Kojima H. et al. 2004), an *in vitro* study using human serum noted that malathion displaces estrogens and androgens from sex hormone-binding globulin (SHBG), a transport protein to which they normally bind in blood. Alterations of this activity "cause dramatic changes in the free concentrations" of these hormones (Meulenberg E. 2002).

DNA damage and altered gene expression following malathion exposure have been observed in several species that may indirectly impact endocrine function. *In vivo* and *in vitro* studies show that malathion can elicit chromosomal aberrations, sister-chromatid exchange as well as an increase of micronuclei frequency in human lymphocytes (Blasiak J. et al. 1999; Windham G. et al. 1998). Malathion is also considered a reproductive cytotoxicant in earthworms, causing DNA fragmentation generally at dose ranges (between 80 and 600 mg/kg^{-1}) soil far above plausible environmental exposure scenarios (Espinoza-Navarro O. and Bustos-Obregon E. 2005). In mice, malathion exposure decreases oocyte viability by 52 percent following 24 hour exposure to 250 μM malathion *in vitro*, presumably related to a concurrent downregulation in subunit I of cytochrome oxidase, an enzyme with a pivotal role in the mitochondrial respiratory chain. (Bonilla E. et al. 2008). This effect is echoed in an another *in vitro* study using isolated pig morular embryos, in which decreased expression of genes related to mitochondrial metabolism for both cytochrome subunits I and III were observed (Salazar Z. et al. 2007). Because mitochondrial dysfunction itself leads to impaired oxidative phosphorylation, overproduction of reactive oxygen species, and apoptosis in germ cells and embryonic tissues, malathion may play an important role in developmental or implantational failure (Masoud et al. 2003; May-Panloup et al. 2005; Chen et al. 2006).

There is little consensus on reproductive and developmental NOAELs and LOAELs for malathion exposure effects from these studies and those performed to satisfy regulatory requirements. Data from studies using New Zealand rabbits, Wistar and Sprague-Dawley rats suggest wide ranges for these measures. In rats, reproductive NOAELs between 18.5 and 800 mg/kg/day and LOAELs between 10 and 500 mg/kg/day have been described. Similar data on developmental endpoints suggest a NOAEL at 300 mg/kg/day and LOAELs between 100 and 800 mg/kg/day (Agency for Toxic Substances and Disease Registry. 2003). A separate two-generation reproductive study using Sprague Dawley rats calculated a parental systemic NOAEL of 5,000 ppm (394/451 mg/kg/day in males and females) and LOAEL of 7,500 ppm (612/703 mg/kg/day), based solely on decreases observed in body weight (Schroeder R. 1990). One thing that is consistent is that all of these NOAELs and LOAELs are relatively high concentrations, well above the range of environmental exposures.

There is also a considerable information base that has identified no significant compound effects on reproductive and developmental measures. In humans, Grether K. et al. (1987) examined an exposed cohort of 24,987 births and an unexposed cohort of 15,278 births and found no biologically significant association between malathion exposure and excess

adverse developmental effects. García A. et al. (1998) compared paternal pesticide exposures between offspring with congenital malformations and controls and found no significant associations with outcomes after adjusting for confounding factors. No significant change in adrenal weights were reported using guinea pigs dermally exposed to 400 mg/kg/day malathion for 30 days (Dikshith T. et al. 1987) and no gross or microscopic lesions were observed in endocrine glands from rabbits treated dermally with up to 1,000 mg/kg/day malathion for three weeks (Moreno O. 1989). While higher doses of 163 mg/kg/day of malathion for seven days damaged the seminiferous tubules in Wistar rats, no significant alterations were noted at 18.5 mg/kg/day, which is far greater than the EPA's estimated environmental concentration (Ojha S. et al. 1992). ***Over all, this information suggests that, while malathion does not directly affect ER or AR receptor binding or activity, exposure at very high doses can alter hormone production and transport that can affect reproductive capacity.***

A. Assessment of estrogenic activity

Many studies have demonstrated a potential degenerative impact of malathion on reproductive function in females, specifically related to toxic effects at the cellular and molecular level of ovarian function in rats. A fifteen day study using Wistar rats intraperitoneally administered up to 33 mg/kg/day showed significantly smaller ovaries with fewer healthy follicles when compared to controls, with a concomitant increase in the number of atretic follicles (Koc N. et al. 2009). Chronic exposure of pregnant rats to malathion and coadministered estrogen demonstrated an increase in cytochrome P450 metabolism of malathion to malaoxon, potentiating overall malathion toxicity by converting a greater proportion of the pesticide active ingredient to the more acutely toxic metabolite malaoxon. Coadministration of progesterone and malathion offered some degree of protection by stimulating hepatic glutathione S-transferase- and carboxylase-mediated detoxification of malathion. In this sense, malathion toxicity is sensitive to estrogen availability and may be impacted by pharmacokinetic changes during pregnancy (Mathews M. and Devi K. 1994; Freuer G. and Kardish R. 1975). Histopathological examination of ovaries from the catfish *Heteropneustes fossilis* exposed to 1.25 mg/L over 96 hours suggest that observed tissue changes reflect a reduction in both serum estrogen levels and follicular cell degeneration (Dutta H. et al. 1994). A fifteen week study using albino rats exposed to up to 100 mg/kg/day, however, showed no changes in ovary histopathology or estradiol 17-beta secretion (Ozmen G. and Akay M. 1993). ***While malathion is capable of affecting estrogen-related processes, evidence suggests that malathion is neither a direct estrogen agonist or antagonist*** (Kojima H. et al. 2004),.

B. Assessment of androgenic activity

Studies of malathion's potential androgenic activity are similarly equivocal (Uzun F. et al. 2009). Malathion-treated rats had significantly lower plasma FSH, LH and testosterone levels than the control rats following 4 week oral dosing study at 27 mg/kg/day. While androgen receptor antagonist substances can suppress FSH and LH by altering the glycosylation of gonadotrophins (Naz R. 1999), malathion may reduce these

hormones indirectly by inducing pathological changes in Leydig cells in interstitial tissues. Rats orally administered 50 to 250 mg/kg/day for 60 days showed significant reduction in weights of testes, epididymus, seminal vesicle and ventral prostate in addition to decreased testosterone, testicular and epididymal sperm counts (Choudhary N. et al. 2008). In CHO cells, malathion does not directly activate androgenic receptors (Kojima H. et al. 2004). Other *in vitro* tests have demonstrated that malathion lacks antiandrogenic effects in mouse and human cells (Kitamura S. et al. 2003). It is likely that these effects of malathion relate, at least in part, to the chemical's ability to cross the blood–testis barrier (Uzunhisarcikli M. et al. 2007), after which it may induce oxidative stress and lipid peroxidation that damages testicular membranes. This in turn may cause the degeneration of spermatogenic and Leydig cells, disrupting spermatogenesis and reducing sperm counts while potentially impacting steroidogenesis and androgenic function indirectly (Naz R. 1999). Appearance of malathion-induced histopathological changes including necrosis and edema in the seminiferous tubules and interstitial tissue supports this possibility (Piramanayagam et al. 1996).

C. Assessment of thyroid hormone activity

Twenty-two patients admitted to a regional hospital with organophosphate poisoning presented altered T3, T4 and TSH profiles: two had low T3 (with normal T4 and TSH); two had low T4 (with normal T3 and TSH) and three had low TSH (with normal T3 and T4) levels. Serum levels of these hormones returned to normal values after resolution of poisoning (Güven M et al. 1999). Body weight was not affected in rats during a 21 day oral exposure, although serum concentration of T3 and T4 significantly decreased and TSH secretion significantly increased (Akhtar N. et al. 1996). Additionally, malathion potently inhibits T3 binding to purified Japanese quail transthyretin (qTTR), a major thyroid hormone-binding protein in plasma. By this mechanism, malathion could interfere with thyroid hormone function indirectly, by interfering with TH binding to plasma proteins (rather than intracellular impacts as ligand-binding transcription factors) (Ishihara A. et al. 2003). Since human data exists confirming malathion's potential to modify thyroid hormonal balance, it is unclear what additional useful regulatory information would be gained by further Tier 1 testing.

D. Amphibians and fish

In addition to the reviews taken into consideration by the EPA during reregistration, several recent publications describe the effects of malathion on development and reproduction in amphibians and fish. Malathion suppresses the immune system in northern leopard frogs and Woodhouse's toads following laboratory exposure as well as in frogs collected from pesticide-exposed locations. (Gilbertson M. et al. 2003; and Taylor S. et al. 1999). Malathion also slows the rate of hatching in zebrafish and significantly reduces body length and eye diameters, suggesting teratogenic reproductive effects above 2 mg/L (Cook et al. 2005). The hatching rate was near 100% for malathion concentrations between 0.25 and 2 mg/l, with tail malformations in pro-larvae at all tested concentrations (Fraysse B. et al. 2006). Bonfanti P. et al. (2004) found no inhibition of development in *Xenopus laevis* blastulae at concentrations between 0.375

and 6 mg/l, but malformations were found. Budischak S. et al. (2008) exposed embryos of the pickerel frog, *Rana palustris*, to environmentally realistic concentrations of malathion from 15 to 600 μg/l, finding that malformation frequency increased and both hatching rate and viability decreased as malathion concentrations increased. Because developmental effects in fish and amphibian species (including *Xenopus laevis*) have already been demonstrated following malathion exposure, additional amphibian metamorphosis assays called for in Tier 1 of EDSP would be expected to contribute little or no new information.

E. Birds

In addition to the avian reproductive data considered during reregistration, several studies have more recently examined the impact of malathion on avian populations. Malathion disrupted normal hormone activity and caused genetic damage in several species. In quail, malathion inhibits the binding of thyroid hormone T3 to a protein that normally transports these hormones to their target cells. (Ishihara A. et al. 2003). Malathion administered orally caused genetic damage to chicken bone marrow cells. Damage occurred following a single dose of malathion at all levels tested (Giri S. et al. 2002). Furthermore, western meadowlark populations decreased following malathion treatments for grasshopper eradication programs in five western states. These "declines in bird density likely resulted from reduced food availability" when the insect populations comprising the bulk of the meadowlark's diet collapsed (George T. et al. 1995). Avian populations are clearly impacted physiologically and ecologically by malathion exposure.

III. Summary and recommendations

The existing malathion database contains ample data indicating an array of genotoxic, neurotoxic and teratogenic effects. Indirect endocrine effects have been defined in both sexes among several species that can result in reproductive and developmental effects. ***While additional testing using existing in vitro methods may provide a renewed opportunity to investigate possible mechanisms underlying malathion's putative endocrine effects, the downstream biological outcomes of any endocrine disrupting properties of malathion (e.g. reproductive and developmental effects) are nevertheless associated with dose ranges that EPA has stated are "not relevant to humans" (Environmental Protection Agency. 2009; FIFRA Scientific Advisory Panel. 2000). Malathion has been thoroughly tested in a wide range of vertebrate species using diverse methods, including protocols identical or similar to those required under Tier 1 of the EDSP as well as several tests similar to those proposed for Tier 2, including the rodent two generation reproductive toxicity test as part of registration and reregistration. There is therefore little justification for further testing of malathion as part of the EDSP.***

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Appendix A: Guideline tests performed as part of malathion's 2009 revised RED

OPPTS Guideline	Study Title
Exotoxicity Data	
850.2300	Avian Reproduction
850.1075	Fish Acute Toxicity - Freshwater
850.1010	Acute Aquatic Invertebrate Toxicity
850.1075	Acute Estuarine/Marine Toxicity - Fish
850.1400	Early Life-Stage Fish (Freshwater)
	Early Life-Stage Fish (Marine)
850.1300	Life-Cycle Aquatic Invertebrate
Mammalian Toxicology	
870.1100	Acute Oral - Rat
870.1200	Acute Dermal - Rabbit
870.2400	Primary Eye Irritation - Rabbit
870.2500	Primary Dermal Irritation - Rabbit
870.2600	Dermal Sensitization
870.3200	21-Day Dermal Toxicity - Rat
870.3700	Developmental Toxicity
870.3800	Reproduction and Fertility Effects - 2 Generation Repro
870.4300	Chronic Feeding Toxicity - Rodent Combined Chronic Toxicity/Carcinogenicity
870.4100	Chronic Feeding Toxicity - Non-Rodent (dog)
870.4200	Oncogenicity - Rat
870.5100	Bacterial Reverse Mutation Assay
870.5385	Micronucleus Assay
870.5450	Rodent Dominant Lethal Assay
870.5550	UDS Assay
870.7485	General Metabolism
870.7600	Dermal Absorption
870.7800	Immunotoxicity

Chlorpyrifos CAS number 2921-88-2
Test order numbers EDSP-059101-30 through 35
Test order date: November 5, 2009

I. Introduction

Chlorpyrifos, an organophosphate pesticide (OP), is a widely used insecticide used to control a variety of insects. It was first registered in 1965 for control of foliage and soil-borne insect pests on a variety of food and feed crops. Chlorpyrifos is one of the most widely used organophosphate insecticides in the U.S. and, until 2000 when nearly all residential uses were cancelled, was one of the major insecticides used in residential settings. Currently registered uses include food and feed crops, golf course turf, greenhouses, non-structural wood treatments such as utility poles and fence posts, and as an adult mosquitocide. Structural treatments for termites are also currently registered, but are being phased out. All use of products for structural termite control was prohibited after December 31, 2005. Indoor non-residential uses include shipholds, railroad boxcars, industrial plants and manufacturing plants (EPA 2006).

II. Existing Toxicological Information

EPA's preliminary human health risk assessment for chlorpyrifos indicated dietary (food and drinking water), occupational and residential risk concerns. The revised risk assessment indicates that, with implementation of the June 2000 mitigation agreement, dietary risks from food are not of concern. Drinking water risk estimates based on screening models and monitoring data from both ground and surface water for acute and chronic exposures are generally not of concern. The exception is incidents of contamination resulting from termiticide use, which are highly localized and expected to be declining because the termiticide use is being phased out. There are concerns for some workers who mix, load, and apply chlorpyrifos to agricultural and other non-residential sites (EPA 2006).

a. Interim Reregistration Decision and Follow-Up 2001-2006

In February 2002 the EPA issued an Interim Reregistration Eligibility Decision (IRED) for chlorpyrifos as part of the organophosphate cumulative assessment initiative required by the Food Quality Protection Act (FQPA) of 1996 for the class of organophosphate pesticides (OP) and a Registration Eligibility Decision (RED) was issued in July 2006. According to the RED, "extensive acute and chronic toxicity data are available for chlorpyrifos". The extensive toxicological data are shown below (appendix B from RED 2006). As the IRED was finalized and the review of OPs was completed, the EPA issued a statement on July 31st, 2006 requiring additional testing for two of the OPs reviewed (methidathion and phorate) but no additional data was requested for chlorpyrifos, presumably because it is extremely data rich.

b. Evidence of neurotoxicity

It is well documented that chlorpyrifos affects the central nervous system through cholinesterase inhibition (Gallo 1991). The IRED states:"Effects from chlorpyrifos exposure can cause cholinesterase inhibition in humans; that is, it can over stimulate the nervous system causing nausea, dizziness, confusion, and at very high exposures (e.g., accidents or major spills), respiratory paralysis and death" (EPA 2002). Adult mice were evaluated for behavioral effects of

either fetal and/or neonatal chlorpyrifos exposure at doses not inhibiting fetal and neonatal brain cholinesterase. Chlorpyrifos was given orally at 3 or 6 mg/kg to pregnant females on gestational days 15-18 and offspring were dosed with 1 or 3 mg/kg on postnatal days (PNDs) 1-14. Serum and brain acetylcholinesterase (AChE) activity was evaluated at birth and 24 hours from termination of postnatal treatments. At the highest levels, gestational and postnatal chlorpyrifos exposure affected motor activity in the open field and enhanced synergically agonistic behavior. Postnatal chlorpyrifos exposure increased maternal responsiveness toward pups in females. These findings point to potential neurodevelopmental disorders when exposed to chlorpyrifos but do not clearly indicate estrogenic activity (Ricceri et al 2006).

It is also well documented that OPs inhibit Natural Killer (NK), lymphokine-activated killer (LAK) and cytotoxic T-lymphocyte (CTL) activities by at least the following three mechanisms: 1) OPs impair the granule exocytosis pathway of NK, LAK and CTL cells by inhibiting the activity of granzymes, and by decreasing the intracellular level of perforin, granzyme A and granulysin, which was mediated by inducing degranulation of NK cells and by inhibiting the transcript of mRNA of perforin, granzyme A and granulysin; 2) OPs impair the FasL/Fas pathway of NK, LAK and CTL cells, as investigated by using perforin- knockout mice, in which the granule exocytosis pathway of NK cells does not function and only the FasL/Fas pathway remains functional; 3) OPs induce apoptosis of immune cells" (Li et al 2006).

Data is presented below to address endpoints slated for endocrine disruption activity of Chlorpyrifos. Principal indications of hormone disruption should manifest in abnormalities in mating, gestation, lactation, thyroid organ weight and functionality, significant body weight changes, and sex organ dysfunction.

c. Assessment of estrogenic activity

No effects on reproduction occurred in a ***three-generation study with rats*** fed dietary doses as high as 1 mg/kg/day (ACGIH 1986). Additionally, no birth effects were seen in the offspring of male and female rats fed 1mg/kg/day during a three generation reproduction and fertility study (ACGIH 1986). *In vitro* tests were conducted to determine estrogen-like effects on ***MCF-7 cell proliferation and effects on CYP19 aromatase activity in human placental microsomes. Chlorpyrifos induced a weak response in estrogenicity assays.*** (Andersen et al 2002). Overall, there is little evidence of estrogenic activity (both *in vitro* and *in vivo*) for chlorpyrifos.

d. Assessment of androgenic activity

A recent studied in rat exposed orally for 30 days showed an dose-responsive effect of chlorpyrifos on testes size, sperm count, and circulating testosterone levels, and decreased fertility. at the dose levels of 7.5, 12.5 and 17.5 mg/kg/day was administered orally to male rats of Wistar strain for 30 days. The body weight of animals did not show any significant change, however, a significant reduction was observed in testes (Joshi et al. 2007). Given the information from this study, a further Hershberger study would be redundant.

e. Assessment of thyroid modulating activity

In ***a two- generation study***, CD1 mice were examined to determine the potential short- and long-term effects of chlorpyrifos on thyroid and adrenal glands following exposure at dose levels not

inducing brain acetyl cholinesterase (AchE) inhibition, during gestational and/or postnatal vulnerable phases. Pregnant dams were treated with 0, 3, 6 mg/kg/day on gestational days 15-18. After delivery, pups were treated subcutaneously on postnatal days (PND) 11-14 with: 0, 1, 3 mg/kg/day of chlorpyrifos. Serum thyroxin (T4), thyroid and adrenals histology and histomorphometry were evaluated in dams and in F1 mice. In dams at 6 mg/kg, decreased T4 levels and increased cell height in thyroid were observed (DeAngelis 2009). Thyroid cell height changes can be explained as an effect of the decreased T4 levels but cell heights should also be accompanied by an increase in thyroid size which is not seen in this study.

f. Fish and birds

Influence of body size on inhibition of brain acetylcholinesterase (AChE) activity of juvenile Nile tilapia, *Oreochromis niloticus* by chlorpyrifos was investigated concerning its potential use in the biomonitoring of anticholinesterase pesticides in tropical water bodies. Three size groups of fish (fry: 3-4 cm, fingerlings: 6-8 cm, sub-adults: 10-12 cm in total length) were exposed to a series of concentrations of chlorpyrifos (0.5-12 ug/L) and concentration-response for inhibition and recovery of the AChE enzyme was evaluated in comparison to the controls at different time points, 2, 6, 10, and 14 days. In the control groups, AChE activity was nearly twice a high for the fry as for the sub-adults. Following 48 hours of pesticide exposure, the AChE activity of the three size groups of fish decreased significantly in comparison to the respective controls. The activity was greatly inhibited in the fry (39-85%) (Chandrasekara 2007). The researchers concluded that body size (e.g. stage of development) determined the extent of AChE inhibition and smaller (younger) individuals were most vulnerable. In another study using the same fish species, researchers found that chlorpyrifos affected cholinesterase (ChE) but that neither gender nor developmental maturity had any significant effect on the body size specific ChE activities. (Pathiratne et al 2008). Smaller fish tended to have larger amounts of ChE and the higher the ChE, the greater percentage of reduction when exposed to chlorpyrifos. Indeed, the mode of action is the same for fishes as in mammals.

Two ***one-generation reproductive studies*** resulted in a NOEL of 125 ppm (the highest dose tested) for bobwhite quail (Fink et al 1978). In another ***one generation reproductive study***, there was no evidence of changes in weight gain, or in the number, weight and quality of eggs produced by hens fed dietary levels of 50 ppm, or about 5.12 mg/kg, of chlorpyrifos (Hayes 1982). Using the oral LD50 level of chlorpyrifos (10.79 mg/kg) chicks showed clinical signs of cholinergic toxicosis within 2 hours of dosing and reduced plasma ChE (Mohammad 2008). These studies indicate that chlorpyrifos works similarly via ChE in birds as it does in other species and that it does not appear to cause adverse effects on reproduction in birds.

III. That Conclusions and Recommendations

Chlorpyrifos has been actively studies for a spectrum of toxicities as well as mechanisms of action for more than 50 years. Data is abundant and clearly signify a neurological effect of inhibition of cholinesterase in humans, fish, rodents, and birds. Affects on reproduction in both males and females has been documented, although the evidence suggests these affects are not mediated via the ER, AR or thyroid hormone pathways. There are already abundant and adequate data for regulating chlorpyrifos, and there is little justification for further analysis via the vertebrate animal tests required by the EDSP. If the EPA requires further information regarding

mechanism of action, this is best pursued in a series of *in vitro* tests such as those being characterized in the ToxCast® program.

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