January 17, 2011

Dr. William Wooge
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Environmental Protection Agency
1200 Pennsylvania Ave. N.W.
Washington, DC 20460-0001

Re: 75 FR 70248, November 17, 2010, Docket ID number EPA-HQ-OPPT-2009-0477; Endocrine Disruptor Screening Program; Second List of Chemicals for Tier 1 Screening.

Dear Dr. Wooge:

These comments are submitted on behalf of the more than two million members of Alternatives Research and Development Foundation, the American Anti-Vivisection Society, the People for the Ethical Treatment of Animals and the Physicians Committee for Responsible Medicine who share the common goal of promoting reliable and relevant regulatory testing methods and strategies that protect human health and the environment while reducing, and ultimately eliminating, the use of animals. These comments are in response to the Environmental Protection Agency’s (EPA) issuance of the second list of chemicals to be tested in the EPA’s Endocrine Disruptor Screening Program (EDSP).

The authority EPA is citing for issuing test orders for these chemicals is not only the Federal Food, Drug and Cosmetics Act (FFDCA) that created the EDSP and under which the test orders for the first list of chemicals were issued, but also the Safe Drinking Water Act (SDWA) and House Appropriations Committee report language for Fiscal Year (FY) 2010. The FFDCA allows testing of chemicals other than pesticides “that may have an effect that is cumulative to an effect of a pesticide chemical if the Administrator determines that a substantial population may be exposed.” In addition, the SDWA authorizes EPA to test “any other substance that may be found in sources of drinking water if the Administrator determines that a substantial population may be exposed…” (emphasis added).

The House report language (U.S. Congress 2009) (which is non-binding) instructs EPA to “publish within one year of enactment a second list of no less than 100 chemicals for screening that includes drinking water contaminants, such as halogenated organic chemicals, dioxins, flame retardants (PBDEs, PCBs, PFCs), plastics (BPA), pharmaceuticals and personal care products, and issue 25 orders per year for the testing of these chemicals. This process also should allow for public input.”

The House report language also instructs EPA to “engage in a timely re-evaluation of the battery of screening, replacing outdated ones with updated, more efficient screens that have been validated (for example, a recombinant receptor assay to replace the cytosolic receptor assay for estrogen receptor binding)” and “develop and publish criteria for evaluating the results of Tier I screening and determining whether a chemical should undergo Tier II analysis within one year of enactment,” neither of which the EPA has done.
The chemicals on the second list were selected from those designated as priority chemicals by either the Office of Water or the Office of Pesticide Programs. EPA began with drinking water contaminants, both regulated (85 chemicals), and unregulated contaminants listed on the third Contaminant Candidate List (CCL 3) (116 chemicals). EPA has determined that CCL 3 chemicals “may occur in sources of drinking water,” and that “a substantial population may be exposed to such substance…” (emphases added) (EPA 2010a).

EPA stated that election of pesticides was based on those that were scheduled for Registration Review during FY 2007 and 2008 (59 chemicals). From this potential list of 260 chemicals, 113 were selected for inclusion in the second list of potential chemicals for EDSP screening.

Screening all 113 chemicals in the battery of 11 Tier 1 tests would kill more than 67,000 animals and cost anywhere from $53 to $144 million. Justification thus far provided for the need and use of endocrine related information does not support a testing program of this magnitude. Our comments present information available for a subset of the chemicals on the second list that would preclude the need for testing those chemicals in the EDSP Tier 1 assays.

Exemptions

In this FR notice, EPA cites only one general exemption, the exemption from FFDCA for substances that are “anticipated not to produce any effect in humans similar to an effect produced by a naturally occurring estrogen.” (21 U.S.C. 346 a(p)(4)). In contrast, the description of the first list of chemicals contained an expanded and clarified exemption, excluding “substances anticipated to have low potential to cause endocrine disruption (e.g., certain Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) List 4 inerts, most polymers with number average molecular weight greater than 1,000 daltons, strong mineral acids, and strong mineral bases).” EPA also excluded from the first list “‘positive control’ substances that are being used by EPA to validate screening assays proposed for the Tier 1 battery” and “chemicals that are no longer produced or used in the U.S.” The draft supporting statement for the Information Collection Request that applies to this Phase I chemical list describes a potential response to testing orders as “Additional EDSP screening is unnecessary because the chemical is an endocrine disruptor or was used as a “positive control” in the EDSP validation effort” (EPA 2010b). These additional exemptions should be listed here as well.

During the selection of chemicals for the second list, the list of potential chemicals was “streamlined” by excluding: 1) biological and naturally occurring substances, 2) chemicals for which manufacturer, importer or registrant cannot be identified, 3) chemicals on first EDSP list, 4) hormones with confirmed endocrine effects, 5) chemicals not likely to be biologically active or have properties incompatible with testing, and 6) pesticides scheduled for registration review after FY 2008.

Many of the chemicals on this list have significant existing information that would preclude further testing. In addition, several are no longer in production or use in the U.S., and therefore mitigation by restricting use is not possible. For these chemicals there is also the question of whether a substantial population is, in fact, exposed, and who would be
responsible for testing (i.e., who would receive the test orders). Rather than issuing blanket test orders for this extensive list of chemicals, existing toxicological and exposure information should be evaluated to determine the regulatory need for information regarding endocrine disrupting potential prior to consideration of further testing.

EPA has begun using (Q)SAR and read-across to bolster information on chemicals under the purview of its pesticides program, after using similar strategies in the “toxics” program for decades. In its proposed rule to revise Part 158W [antimicrobial pesticide] testing requirements, it stated its interest in using QSAR and read-across to fill data gaps where appropriate (EPA 2008a). Furthermore, within the High Production Volume (HPV) Challenge Program and the Chemical Assessment and Management Program (ChAMP), EPA has had relatively good success utilizing categorization and read-across to cut down on the amount of testing (and thus animals and time used) while assessing large numbers of chemicals at once. Indeed, a few of the substances on this second list were tested under the HPV challenge (1,2,4-trichlorobenzene and 1,2-dichloropropane). We urge the agency to use QSAR and read-across technology and concepts for easily grouped candidate chemicals—such as the organochlorides—instead of ordering testing for each substance for each assay.

I. Pesticides

As pointed out in comments regarding the first list of chemicals (People for the Ethical Treatment of Animals (PETA) et al. 2008), pesticides are among the most highly data-rich substances in existence. For registration, pesticides currently are subject to dozens of separate animal tests, including reproductive and chronic/lifecycle studies in rodents, fish and birds (EPA 2007a). These tests kill thousands of animals and include many of the same endpoints addressed in the presumptive EDSP Tier 2 tests. Similarly, EPA’s HPV Program also provides for the collection of data which may be germane to the assessment of potential reproductive toxicity (EPA 2000a). At an absolute minimum, chemicals should be exempted from EDSP Tier 1 screens for which equivalent or higher tier data are available.

In its guidance on the acceptance of Other Scientifically Relevant Information (OSRI) in lieu of additional testing, EPA describes OSRI as any information that “informs the determination as to whether the substance may have an effect that is similar to an effect produced by a substance that interacts with the estrogen, androgen, and/or thyroid hormonal systems. OSRI may either be functionally equivalent to information obtained from the Tier 1 assays—that is, data from assays that perform the same function as EDSP Tier 1 assays—or may include data that provide information on a potential consequence or effect that could be due to effects on the estrogen, androgen or thyroid systems,” including “studies that correspond to the assays in Tier 1 or Tier 2; metabolism studies; or other vertebrate or invertebrate toxicity studies, including, but not limited to, developmental toxicity tests, carcinogenicity tests, toxicogenomic data, and reproductive toxicity tests”, as well as testing conducted under 40 CFR Part 158 data requirements to support a pesticide registration (EPA 2009a).

Following this reasoning, information requests should be tailored to individual chemicals and/or chemical classes. For example, Reproduction and Fertility effects (OPPTS 870.3880) and Prenatal Developmental Toxicity (OPPTS 870.3700) tests are required for both food-use and non-
food-use pesticide Technical Grade of the Active Ingredients (TGAI). The simple mechanistic
data produced by the Hershberger, uterotrophic, and the male and female pubertal assays will not
provide additional information; indeed, chemicals tested according to OPPTS 870.3880 have, in
effect, already been subject to EDSP Tier 2 mammalian testing. Thus, with the possible
exception of mechanistic screening for thyroid effects, EDSP Tier 1 screens would appear to
provide little or no value-added for pesticide chemicals.

Alachlor

Alachlor is a chloroacetanilide herbicide used primarily for pre-emergent control of annual
grasses and broadleaf weeds in corn, sorghum, soybeans and other crops. The annual use of
alachlor on corn has been reduced substantially with the registration of acetochlor in 1994
(National Agricultural Statistics Service (NASS) 2010). As there are no residential uses for
alachlor, public exposure would be mainly through food and drink (EPA 2006a). Tolerances for
residues on a variety of foods have been set by EPA (40 CFR §180.249) and residues of alachlor
in food are usually below the detection limit (WHO 2003a). EPA has assessed the chronic (non-
carcinogenic) dietary risk posed by alachlor, and determined that the Anticipated Residue
Concentration (ARC) for the overall U.S. population represents less than 1% of the chronic
Reference Dose (RfD), the amount believed not to cause adverse effects if consumed daily over a
70-year lifetime (EPA 1998). Levels of parent alachlor were measured in a long-term
groundwater monitoring study of vulnerable areas, which found detectable levels in less than 1% of
the 10,054 samples collected (De Guzman, Hendley et al. 2005). In a seven-year surface water
drinking supply monitoring study conducted in a 12-state area in mostly vulnerable watersheds,
parent alachlor was detected in 7% of the samples and annualized mean concentrations steadily
decreased from about 0.015 ppb in 1995 to <0.005 ppb in 2001 (Hackett, Gustafson et al. 2005),
and rarely, if ever, exceeded the maximum contaminant level (MCL) of 2 ppb alachlor in drinking
water as set by EPA (EPA 1998).

EPA reports short-term exposure to alachlor can lead to eye and skin irritation, while long-term
exposure may result in damage to liver, kidney, spleen, the lining of the nose and eyelids, and
cause cancer (EPA 2010c). Studies to determine actions on the endocrine system have produced
results that are variable and inconclusive.

In an ER competitive-binding assay using rat uterine cytosol, alachlor did not exhibit any effect
on [³H]17β-estradiol at a 50% inhibition concentration (IC₅₀) >1000 µM (Blair, Fang et al. 2000).
Klotz et al. (1996) utilized three assays, yeast estrogen screen (YES), competition binding with
the human estrogen receptor (hER), and activation of an estrogen-responsive reporter gene in
MCR-7 human breast cancer cells, to test a variety of chemicals (Klotz, Beckman et al. 1996).
They reported weak activity by alachlor in the YES at a concentration of 10 µM, weak binding
inhibition of [³H]17β-estradiol (E2) to (hER) at relatively high concentrations of ≥100 µM, and
moderate induction of luciferase activity in breast cancer cells at 1 µM, which did not further
increase at 10 µM, indicating that alachlor may be only a partial agonist of hER. However, Soto
et al. (1995) showed that alachlor in similar concentrations was not estrogenic in the E-SCREEN
assay, a sensitive test that evaluates proliferation of MCF-7 human breast cancer cells under the
influence of estrogens (Soto, Sonnenschein et al. 1995). Nor did alachlor show activity in trout
reporter gene and vitellogenin production assays (Petit, Le Goff et al. 1997). In a study to
determine the ability of chemicals to bind the alligator estrogen receptor (aER) and progesterone receptor (aPR) using a protein extract prepared from the oviduct of the alligator, Vonier et al. (1996) reported an IC\textsubscript{50} of 27 µM for alachlor as compared to 0.0078 µM for 17ß-estradiol, but no interaction by alachlor with the aPR (Vonier, Crain et al. 1996). Jin et al. (1997) demonstrated neither agonist nor antagonist activity of alachlor in a recombinant human progesterone receptor (hPR) expressed in yeast cells (Jin, Tran et al. 1997). Kojima et al. (2004) tested 200 pesticides for both agonism and antagonism to two hER subtypes, hER\textalpha and hER\textbeta, and a human androgen receptor (hAR), via transactivation assays using Chinese hamster ovary cells (Kojima, Katsura et al. 2004). Alachlor showed antiestrogenic properties in the hER\textalpha transactivation assay with 10\textsuperscript{10} M E\textsubscript{2}, but at a significantly higher dose (10\textsuperscript{-5} M) than the well-known ER antagonist tamoxifen at 10\textsuperscript{-8} and 10\textsuperscript{-7} M. Assessment by Kojima et al. (2004) of these same pesticides for their inhibitory effect on androgenic activity showed alachlor to be a weak inhibitor, with an RIC\textsubscript{20} (concentration of the test compound showing 20% inhibition of the androgenic activity induced by 10\textsuperscript{-10} M DHT) of 9.6 X 10\textsuperscript{-6} M, a considerably higher concentration than the RIC\textsubscript{20} of 4.3 X 10\textsuperscript{-8} M for the diphenyl ether herbicide Chlornitrofen, a potent androgenic inhibitor. Cheek et al. (1999) examined the ability of alachlor to bind a recombinant human thyroid receptor (hTR\textbeta1) and to interact with the serum transport proteins, transthyretin and thyroid-binding globulin (TBG), and found no action at doses as high as 100 µM (Cheek, Kow et al. 1999).

In vivo studies include a three-generation toxicity study in which rats were administered alachlor at dose levels up to 30 mg/kg bw/day (Schroeder and Hogan 1981). No treatment-related effects on mating, fertility, male-female sex ratios, pup survival, testicular weight, or histopathology of the reproductive organs were noted. Developmental toxicity studies conducted by Monsanto in rats and rabbits (Rodwell 1980; Schroeder 1988) revealed no statistically significant differences in the mean numbers of viable fetuses, resorptions, post-implantation losses, total implantations, corpora lutea, sex distribution of pups, or mean fetal body weights between the treated groups and controls. In a rodent carcinogenicity study, male rats developed benign tumors in the thyroid follicular cells at 126 mg/kg/day, but not at 42 mg/kg/day or lower (Wilson, Thake et al. 1996) and the authors concluded that these tumors were produced by way of a non-genotoxic mode of action that includes UDPGT induction, increased TSH, alterations in T3/T4 hormone production and thyroid hyperplasia. Based on excessive body weight loss (>30%), hepatocellular necrosis, and slightly decreased survival, the authors also concluded 126 mg/kg/day was above the MTD. In a risk assessment of the chloroacetanilides alachlor, acetochlor and butachlor, development of thyroid tumors was not a recommended endpoint because, again, toxic effects were noted only at doses above the MTD, and, in addition, humans were more refractory to the induction of thyroid follicular cell tumors than rats (EPA 2006a). An epidemiological study that evaluated >1000 alachlor manufacturing-plant employees showed no evidence of increased incidence of thyroid tumors or any other hormonally-mediated cancer (breast, testicular, ovarian, uterine or prostate) over the 25-year period of record (Acquavella, Riordan et al. 1996). A study of the relationship of hypospadias (a defect of the male reproductive system that has been produced experimentally in animals by prenatal and perinatal administration of pesticides with estrogenic and/or antiandrogenic properties) and proximity to agricultural pesticide use in eastern Arkansas, indicated that alachlor was negatively associated with hypospadias (Meyer, Reif et al. 2006).
In two avian reproductive studies (Gallagher, Beavers et al. 1999a; Gallagher, Beavers et al. 1999b) and a fish reproductive study (Rhodes and Muckerman 1984) any noted effects were generally produced only at the highest doses of alachlor used.

Summary and Conclusions regarding alachlor: Alachlor usage has been steadily declining since the introduction of acetochlor in 1994. Alachlor was detected infrequently in surface and ground waters in vulnerable watersheds where it is used as an agricultural pesticide, and generally at concentrations well below the MCL set by EPA. Tolerances for food residue have been established by EPA and amounts on food are typically below detection limits. This raises the question as to whether a substantial population is, in fact, exposed to alachlor in water and food.

Alachlor appears to have minimal potential to interfere with estrogen-estrogen receptor binding or alter transcriptional activity of estrogen receptor responsive genes. Alachlor does not bind or alter transcriptional activity of the progesterone receptor. Alachlor does not bind to human thyroid receptor, thyroid binding globulin or transthyretin. Production of thyroid tumors in rats occurs only at very high dose levels through a secondary mode of action, i.e., induction of hepatic UDPGT, which has little relevance to humans. Evidence of endocrine disruption was not noted in mammalian toxicity and chronic developmental tests. An epidemiological study of alachlor manufacturing plant employees showed no increase in the incidence of thyroid or any other hormonally mediated cancers. A case-control study relating hypospadias to exposure to agricultural pesticides indicated a negative association for alachlor. No consistent indication of endocrine activity was noted in several bird, fish, and amphibian studies. The lack of likely exposure combined with proven lack of endocrine activity should exempt alachlor from further testing.

II. Pesticides and chemicals no longer registered, manufactured or in use

At least three of the 50 chemicals listed as registered pesticide active ingredients (fenamiphos, pyridate and sulfosate) are not currently registered (EPA 2010c). Hexachlorocyclopentadiene and its derivatives chlordane, endrin, and heptachlor, as well as all other derivatives, have been internationally banned or are under consideration for banning, according to the deliberations of the Stockholm Convention on Persistent Organic Pollutants (Stockholm Convention 2008). In addition, many more of SDWA regulated contaminants and CCL3 chemicals are no longer manufactured. For chemicals that are no longer manufactured, it is unclear who would perform the proposed testing. More importantly, since the purpose of the information from the EDSP is presumably to mitigate exposure to dangerous chemicals, it is unclear what EPA intends to do with additional information about chemicals that are no longer in production. In addition, there is already significant information regarding exposure, toxicity and potential endocrine activity for many of these chemicals including the pesticides that are no longer registered. Therefore we question the justification and utility of including these chemicals in Tier 1 screening and request that they be exempted.
A. Fenamiphos

Fenamiphos is an organophosphate, one of a group of related pesticides that affect the functioning of the nervous system. A nematicide and an insecticide, fenamiphos was used primarily to control nematodes and thrips on various agricultural crops (i.e., citrus, grapes, peanuts, pineapples, tobacco, etc.) and non-agricultural (i.e., turf and ornamentals) sites. Additionally, all uses were soil incorporated, except for the pineapple use. There were no residential uses for fenamiphos.

In the Interim Reregistration Eligibility Decision (IRED) for fenamiphos (EPA 2002), EPA determined that due to a number of unacceptable risk levels identified, the registrant would need to develop risk mitigation measures if it wished to continue the pesticide’s registration. Instead, Bayer Corporation, the sole registrant, requested voluntary cancellation and a 5-year phase-out of all existing fenamiphos registrations rather than committing to develop any additional data. A Use Deletion and Product Cancellation Order (FRL-7332-5) (68 FR 68901) for fenamiphos was published in the Federal Register on December 10, 2003, announcing that EPA granted the registrant’s request. All fenamiphos product registrations were cancelled and sale and distribution of manufacturing and end-use products by the registrant were prohibited effective May 31, 2007.

Sale and distribution of all existing stocks by persons other than the registrant were to be prohibited effective May 31, 2008. However, as announced in an Amendment to Use Deletion and Product Cancellation Order published in the Federal Register on June 11, 2008 (FRL-8368-2), persons other than the registrant were allowed to continue to sell and distribute two fenamiphos products, Nemacur 10% Turf and Ornamental Nematicide (EPA Reg. No. 432-1291) and Nemacur 3 Emulsifiable Systemic Insecticide-Nematicide (EPA Reg. No. 264-731) until November 30, 2008. A December 2008 amendment (EPA 2008b) provided that persons other than the registrant were permitted to sell and distribute existing stocks of Nemacur 3 Emulsifiable Systemic Insecticide-Nematicide (EPA Reg. No. 264–731) until March 31, 2009. Tolerances in 40 CFR 180.349 for most commodities were revoked by December 31, 2009, with the exception of import tolerances for banana; fruit, citrus, group 10; garlic; grape; and pineapple, which will not have a U.S. registration as of December 31, 2009. On July 23, 2009, a petition from the American Bird Conservancy was submitted, requesting that EPA revoke tolerances established under the Federal Food, Drug and Cosmetic Act (FFDCA), section 408, for uses of 13 pesticides with no associated U.S. registrations (including fenamiphos), claiming that maintaining these tolerances allows Central and South American countries to continue using these pesticides on crops for which the United States has already determined there are unacceptable risks for U.S. protected migratory birds (EPA 2009b). EPA has yet to take action on this petition.

Summary and conclusions regarding Fenamiphos: Fenamiphos is a canceled pesticide product that has not been produced since 2008 and any remaining stocks could not be sold after March 31, 2009. Tolerances on nearly all commodities were revoked in December 2009; any remaining tolerances will not have a U.S. registration as of December 31, 2009. There is a petition to revoke the remaining few tolerances for imported products. Again, it is questionable whether a substantial population is, in fact, exposed to fenamiphos in water and food. Since fenamiphos is no longer used as a pesticide in the US, and other uses are in question,
information regarding its potential endocrine activity is of little value and this chemical should not be included in the second list of chemicals for screening.

B. Pyridate

On April 18, 2007, EPA issued a notice of receipt of request by registrant Syngenta Crop Protection, Inc. to voluntarily cancel and to terminate uses of pyridate (EPA 2007b). The last remaining pyridate products registered under FIFRA Section 3 were cancelled in 2004 for failure to pay the required annual maintenance fees, but there were several Special Local Needs registrations for weed control on mint that were still active. On November 21, 2007, EPA issued notification in the Federal Register (EPA 2007c) of product cancellation and indicated that any existing stocks if pyridate could be sold and used until exhausted.

Two studies are available that evaluated pyridate along with other pesticides as potential endocrine disruptors. Okubo et al. (2004) examined the estrogenic and antiestrogenic activities of pesticides with suspected residues in vegetables and fruits by using the cell proliferation of estrogen-dependent human breast cancer MCF-7 cells in vitro (Okubo, Yokoyama et al. 2004). Pyridate did not affect cell proliferation at non-cytotoxic concentrations, and was therefore interpreted as having no estrogenic activity. Results of competition by pyridate for binding hER and hAR showed weak affinities (RBA value for ERα of 0.069% as compared to 100% for positive control diethystilbestrol; and RBA value for AR of 0.011% as compared to 100% for positive control mibolerone). The authors concluded that any activity by pyridate was relatively low.

Using a microtiter plate receptor assay with recombinant hAR to evaluate the androgen receptor binding affinity of a number of pesticides, Bauer et al. (2002) reported minimal displacement of [3H]-DHT binding by pyridate at an RBA of 0.007% as compared to DHT at 100% (Bauer, Bitsch et al. 2002). This binding was demonstrated to be reversible in exchange assays.

Summary and conclusions: Pyridate binds weakly to hAR, and hER, but there was no evidence for estrogenic or antiestrogenic properties in cell proliferation assays. While this pesticide was considered to be a potential residue on fruits and vegetables at the time of the Okubo study, its registration has since been canceled and this, as well as any other sources of exposure are no longer of concern. The lack of likely exposure combined with proven lack of endocrine activity should exempt pyridate from further testing.

C. Sulfosate

Sulfosate, a trimethylsulfonium salt of glyphosate, is an organophosphate herbicide that was used as a weed suppressor on a variety of commercially important crops. All sulfosate registered products were canceled on October 15, 2004 due to non-payment of registration maintenance fees, and a notice was published in the Federal Register on October 27, 2004 titled “Cancellation of pesticides for non-payment of Year 2004 maintenance fees” (EPA 2004a). EPA stated that the registrant (Zeneca Ag. Products, now Sygenta Crop Protection) could continue to sell and distribute existing stocks until January 15, 2005; however the registrant later informed EPA that it did not produce sulfosate after 2002 and sold the remaining existing stocks of sulfosate in 2003
Also, the registrant did not support continuing the import tolerance on bananas. Therefore, by 2004 EPA believed all existing stocks had been exhausted and any treated commodities had cleared the channels of trade. On May 2, 2007, EPA proposed amendments to revoke sulfoxafile’s existing tolerances in 40 CFR 180.489 (EPA 2007d), and finalized the revocation, after a 60-day comment period in which no comments were received, on September 19, 2007 (EPA 2007e).

Summary and conclusions: Sulfosate is a canceled pesticide product that has not been produced since 2002 and the last stocks were sold in 2003. Tolerances on all commodities have been revoked. Since it is unlikely that a substantial population is exposed, information regarding the potential endocrine activity of this pesticide is of questionable value and this chemical should not be included in the second list of chemicals for screening.

D. Lindane

Similarly, lindane is no longer registered as an insecticide and has been internationally banned from agricultural use since 2009 under the Stockholm Convention on Persistent Organic Pollutants (EPA 2006b; Engeler 2009). Although lindane is still allowed sparingly as a second-line treatment for lice and scabies (Engeler 2009), it has not been manufactured in the US since the 1970s (Commission for Environmental Cooperation (CEC) 2006). As the agricultural use of lindane was restricted and eventually ceased, the levels of this substance in the environment and in human tissue have declined dramatically. Consistent with the calculated half-life of lindane isomers in human blood of 7.2 years (Hines 1992), as early as 2005 no detectable amounts of lindane could be found in a random sampling of about 5,000 people in the US (Centers for Disease Control and Prevention (CDC) 2005) and the amount of lindane introduced into the environment has been reduced by more than 99% since agricultural restrictions have been in place (Commission for Environmental Cooperation (CEC) 2006; EPA 2006c). State-specific bans on the pharmaceutical use of lindane and international efforts to discontinue lindane production are reducing the environmental load even further (Humphreys, Janssen et al. 2008). According to the Toxics Release Inventory, the remaining lindane waste in the US resides in a very limited number of hazardous waste recovery facilities that reported only 236 lbs of controlled lindane discharge in 2009. This minimal release would not result in a substantial population being exposed.

As with the other pesticides mentioned above, there is no clear entity to whom testing orders would be issued for lindane and additional data would not alter mitigation strategies already successfully underway. These aspects alone should exempt lindane from testing in this program. In addition, considerable data have already been collected on the endocrine disrupting potential of lindane, its isomers and its byproducts. The organochlorines, the chemical class to which lindane belongs, are a particularly data rich class of compounds that have been shown to act as competitive inhibitors of androgen receptors and to induce conversion of androgen to oestrogen in a variety of systems (Sonnenschein and Soto 1998; Daxenberger 2002; Lemaire, Terouanne et al. 2004; Scippo, Argiris et al. 2004; Storrs and Kiesecker 2004; McKinlay, Plant et al. 2008).

A large number of experiments on animals have shown that lindane exposure can lead to reproductive toxicity in animals through a variety of mechanisms including disruption of steroid
hormone homeostasis, hormone receptor binding and disruption of spermatogenesis (McKinlay, Plant et al. 2008; Di Consiglio, De Angelis et al. 2009). Tests on animals have also indicated that lindane exposure can shorten oestrous cycles, lower luteal progesterone concentrations, increase blood serum concentrations of insulin and oestradiol and alter thyroid hormone levels in the animals used (Rawlings, Cook et al. 1998; Beard and Rawlings 1999; Alvarez-Pedrerol, Ribas-Fito et al. 2008). Evidence for lindane's estrogenic (Raizada, Misra et al. 1980; La Sala, Farini et al. 2010) and anti-estrogenic activities (Chadwick, Cooper et al. 1988; Cooper, Chadwick et al. 1989) is so widely accepted that it has been used as a control to validate in vitro estrogenic assays (Scippo, Argiris et al. 2004; La Sala, Farini et al. 2010). In addition, several epidemiological studies have linked lindane to infertility and premature births in humans (Colborn, vom Saal et al. 1993; Cioroiu, Tarcau et al. 2010).

Summary and conclusions: Lindane is a regulated drinking water contaminant that has been banned from use as a pesticide and as a result, exposure is decreasing rapidly. Lindane has already been shown to have endocrine-disrupting activity that results in reproductive toxicity; therefore additional testing would be redundant and is not warranted.

E. 2-Dibromo-3-Chloropropane (DBCP) and 2,4,5-TP

DBCP was banned as a pesticide on pineapple by the EPA in 1977 because of its known neurotoxicity and probable human carcinogenicity. In 1985 EPA cancelled all registrations and prohibited use of any stockpiles of DBCP (EPA 2000b). Although DBCP has been detected in ground water, filtration systems have been developed that can remove it from drinking water sources making human exposure to residual DBCP unlikely and easily mitigated (BCERF Program on Breast Cancer and Environmental Risk Factors 2004). Similarly, the herbicide 2,4,5-TP has been banned in the US since 1985. According to the EPA's own fact sheet, the physical properties of 2,4,5-TP make it very unlikely to leach into ground water and to accumulate in aquatic life. Even so, levels of 2,4,5-TP in drinking water are currently monitored and in the case of detection above action levels, would be easily removed with activated charcoal (EPA 2010d).

Summary and conclusion: DBCP has not been used in the US for over 30 years; the only possible mitigation is by removal from drinking water supplies by filtration. Therefore information on endocrine activity of this chemical is not germane to its regulation and it should be removed from the second list for testing.

F. Dinoseb

Due to several poisoning cases and deaths of manufacturing workers attributed to this herbicide, it was banned by the EPA in 1986. Despite significant use, well water contamination at the time was found to be limited to two low-use wells. The ban was partially due to substantial evidence for this herbicide's endocrine disrupting properties, although there is currently very little potential for human exposure to dinoseb. Studies have reported birth defects in animals born to females fed comparatively low doses as well as abnormal sperm and decreased weight of the thyroid gland in males at doses close to calculated occupational exposures at the time (Felsot 1998).
Summary and conclusions: There is clear indication that dinoseb interferes with reproduction and development there is currently very little chance of exposure, therefore Tier 1 information would not be useful in regulation of this substance and it should be removed from the second list of chemicals for screening.

G. Carbon tetrachloride

Carbon tetrachloride (CCl₄) is an established human hepatotoxin and nephrotoxin whose metabolites cause oxidative tissue damage that leads to the development of steatosis, necrosis and cirrhosis of the liver in rats (Ruprah, Mant et al. 1985; Abraham, Wilfred et al. 1999; Bruckner, Ramanathan et al. 2002; Dang, Wang et al. 2007). Specifically, the chemical is activated by CYP2E1 to form the trichloromethyl radical (CCl₃•), which can variously bind to cellular molecules leading to the fatty depositions characteristic of steatosis or, in the case of CCl₃•-DNA adducts, hepatic cancer (Weber, Boll et al. 2003). As a result, toxicity due to CCl₄ exposure can be interrupted with the use of antioxidants and mitogens (Weber, Boll et al. 2003). As outlined in Title 40 of the Code of Federal Regulations part 82, CCl₄ production and importation has been banned in the United States since 1996 because of its documented toxicity.

Because of the enzyme’s role in mediating metabolic activation, tissues with high relative expression of CYP2E1, including the liver and kidneys, are especially vulnerable to CCl₄-induced toxicity (ATSDR 2003). Because CYP2E1 is also present in testicular tissues, the chemical impacts the spermatogenic cycle in rats, ultimately leading to hypogonadism similar to that seen in human patients with alcohol-induced cirrhosis (Van Thiel, Gavaler et al. 1980; Sundari, Wilfred et al. 1997; Abraham, Wilfred et al. 1999; Horn, Ramos et al. 2006). Rats exposed to 2 ml CCl₄/kg/week for 16 weeks showed decreased testicular weights relative to controls, in addition to abnormal testicular histopathologies including complete atrophy of seminiferous tubules and germ cell degeneration (Khan and Ahmed 2009). In the same animals, CCl₄ exposure was associated with a dose-dependent decrease in serum testosterone, follicle stimulating hormone and luteinizing hormone, likely due to oxidative damage to Leydig cells (Santos, Ferraz et al. 2004; Khan and Ahmed 2009).

Summary and conclusions: The mechanism of action of this chemical is clearly defined and the toxicity has been well characterized; further testing in EDSP assays would add no regulatory value and is not warranted and CCl₄ should be removed from the second list of chemicals to be tested.

H. Polychlorinated biphenyls (PCBs)

PCBs were used extensively in the manufacture of transformers, capacitors, and other heat transfer devices through the late 1970's. In 1979, their manufacture and importation was banned in the United States, based on mounting evidence that they were toxic to humans and the environment. Extensive exposure and toxicological information is available from the Agency for Toxic Substances and Disease Registry’s (ATSDR) database (Agency for Toxic Substances and Disease Registry (ATSDR) 2000).
PCBs are found throughout the environment. They do not easily break down and bioaccumulate in the fatty tissues of fish and mammals. The major route of human exposure is consumption of fish. Exposure to PCBs is regulated to extremely low levels by several federal agencies. EPA’s standard for PCBs in drinking water is 0.5 parts per billion (ppb) and 0.17 parts per trillion in lakes and streams. FDA’s residue limits for PCBs ranging from 0.2 ppm (parts per million) in infant foods to 3 ppm in poultry and red meat. OSHA regulates worker exposure by inhalation to less than 1 milligram per cubic meter (mg/m³) over a 40-hour work week for 42% chlorine PCBs and to less than 0.5 mg/m³ for 54% chlorine PCBs. PCB levels in tissues from the US population are now declining. In addition, storage and disposal of PCB waste are regulated under the Toxic Substances Control Act (TSCA).

ATSDR reviews existing data on the endocrine effects of PCB exposure and concludes that PCBs can affect a wide variety of endocrine systems. PCBs generally have much lower estrogenic potency than the endogenous hormone, 17β-estradiol. PCB mixtures can produce weak estrogenic responses and PCB congeners with multiple ortho-chlorines may be at least partly responsible for these responses. Estrogenicity in vivo, as measured by increased uterine wet weight, has been observed for Aroclor 1242, PCB 47, PCB 52 and a hydroxylated derivative – but not for the coplanar congener PCB 77. PCB 47 and a hydroxylated derivative also display estrogenic activity in vitro in a cell proliferation assay. In addition, anti-estrogenic effects in vivo have been reported for PCB 77 and in vitro for other congeners with no or single ortho-chlorines.

In humans, PCB exposure may be associated with menstrual disturbances, repeated miscarriages and effects on male fertility. In female rats, reproductive effects of Aroclor 1254 include reduction in preweaning weight gain, delayed puberty, reduced mating rate, reduced implantation rate and mean number of embryos, reduced uterine weight during proestrus, and reduced uterine response to exogenous 17β-estradiol in ovariectomized offspring. Effects in females of various other species have also been described, including decreased conception in mice, partial or total reproductive inhibition in minks, and prolonged menstruation and decreased fertility in monkeys. Female minks and monkeys are particularly sensitive to reproductive effects of PCBs. In male rats, decreases in numbers of normal fertilized eggs, blastocysts, implants and embryos as well as decreases in seminal vesicle and ventral prostate weights have been observed, and gestational and lactational exposure to PCBs can adversely affect morphology and production of sperm and fertility in the male offspring of rats and mice. These data address endpoints evaluated in the ER binding, uterotrophic and Hershberger assays used to identify estrogen and androgen agonists and antagonists in EDSP Tier 1, specifically body weight, uterine weight, vaginal histopathology, ventral prostate and seminal vesicle weights.

Data from animal studies indicate that exposure to PCBs affects thyroid histology, disrupts the production and transport of thyroid hormones and accelerates their metabolic clearance. In particular, neurobehavioral deficits in rat pups exposed to Aroclor 1254 in utero and during nursing were significantly attenuated by subcutaneous injections of T4. Similarly, increased testis weight and sperm production in rats that were administered Aroclor 1254 were attenuated by injections of T4. Similar effects to those produced by PCBs can be produced by other hypothyroid-inducing agents.
These data address endpoints evaluated in the pubertal male and female assays used to identify anti-thyroid activity in EDSP Tier 1. In addition, other effects of PCBs on endocrine function are noted in animal studies including effects on the adrenal glands and serum adrenal steroid levels.

PCBs have induced endocrine-related effects in a variety of other taxa. For example, fish in early life stages were more vulnerable than adults to PCB toxicity. Avian reproduction was impaired due to decreased egg hatchability and increased embryotoxicity. A suite of developmental effects observed repeatedly in Great Lakes wildlife has been characterized as the Great Lakes embryo mortality, edema, and deformity syndrome (GLEMEDs syndrome).

Summary and Conclusions: PCBs are an extremely well-studied chemical class; their endocrine disrupting potential has been demonstrated in rodents, birds, fish and other wildlife as well as in epidemiologic studies, including information for several of the Tier 1 assay endpoints. Considering this toxicological information as well as the fact that the production and use of PCBs in nearly all circumstances have been banned for over thirty years, that exposure to remaining PCBs is already tightly controlled by existing regulations and that remediation of PCB contaminated sites is also subject to TSCA regulation and EPA guidance, it is highly unlikely that any new information would be gained from further testing in the EDSP Tier 1 assays or that any such information would result in additional regulatory action. It is more likely that risk reduction will be achieved through continued emphasis on clean-up and revitalization.

III. Substances used in Tier 1 assay validation

Three of the Phase II chemicals (methoxychlor, fenarimol and vinclozolin) were used in validation studies for Tier 1 assays. Together with the abundance of other information regarding the endocrine activity of these chemicals, these data render further testing redundant and there is therefore no reason for including these chemicals in the second list for screening.

A. Methoxychlor

Methoxychlor has weak ER agonist and AR antagonist activity and was used as a reference chemical in validation studies for the AR binding, female pubertal, male pubertal, and uterotrophic assays (EPA 2007f). In the female pubertal experiment, methoxychlor advanced the onset of vaginal opening, affected estrous cyclicity “as expected,” and decreased serum levels of TSH and T4 hormones (EPA 2007g). In the uterotrophic validation studies, methoxychlor weakly affected uterine weight gains (Organisation for Economic Co-operation and Development (OECD) 2003). In an analysis of ToxCast information with particular focus on endocrine activity, methoxychlor and its more active metabolite, 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), were used as reference chemicals, based on their well-characterized estrogenic activity (Reif, Martin et al. 2010). Both chemicals showed expected high activity in multiple estrogen binding and transcriptional activation assays. These chemicals both have estrogenic effects (Miller, Gupta et al. 2006), and in ToxCast data, the daughter
metabolite (HPTE) demonstrated higher potency across more assays than its parent, consistent
with evidence showing that the metabolism of methoxychlor to HPTE results in higher ER
affinity (Nimrod and Benson 1997). In contrast, when tested in a male pubertal assay, methoxychlor did not effect preputial separation or thyroid levels, and no treatment-related histopathology was noted (EPA 2007h).

In addition to the extensive information available on its potential endocrine activity, methoxychlor was banned in the United States in 2003 (EPA 2004b) and in the European Union in 2002 (European Union - DG SANCO 2002). Although these bans are comparatively recent, methoxychlor biodegrades relatively quickly in the environment and does not bioaccumulate (Smith 1991; Wauchope, Buttler et al. 1992) making it very unlikely that a substantial population would be exposed to levels of concern.

B. Fenarimol

In validation studies for the female pubertal and aromatase assays, fenarimol was used as a weak aromatase inhibitor control (Battelle, Brueggemeier et al. 2007; EPA 2007g). Fenarimol has also demonstrated estrogenic activity in an uterotrophic assay (Andersen, Bonefeld-Jorgensen et al. 2006) and anti-androgenic activity in a Hershberger assay (Vinggaard, Jacobsen et al. 2005). In addition to in vitro and mammalian studies, fenarimol has been tested in echinoderms (Sugni, Tremolada et al. 2010), shrimp (Kumar, Correll et al. 2010), and daphnia (Hassold and Backhaus 2009). In fathead minnows, fenarimol reduces plasma vitellogenin and egg numbers in female fish (Thorpe, Benstead et al. 2007).

C. Vinclozolin

Vinclozolin is a well characterized anti-androgenic fungicide known to inhibit testosterone binding (Kelce, Monosson et al. 1994) and was used as a control in validation of the fish short-term reproduction, Hershberger, steroidogenesis, and male pubertal assays. In validation studies for the fish short term reproductive assay, vinclozolin caused significant decreases in fecundity at high doses (900µg/L). Male secondary sex characteristics, testicular degeneration, and male gonad weight were the most sensitive endpoints (EPA 2007i). In validation studies of the Hershberger assay, vinclozolin caused decreased weights of all five male sex accessory tissues (Organisation for Economic Co-operation and Development (OECD) 2004). In validation studies of the steroidogenesis assay, vinclozolin increased estradiol and decreased progesterone (M.H., M.M. et al. 2005). In validation studies of the male pubertal assay, vinclozolin caused delayed preputial separation and increased body weight at puberty (EPA 2007h). Vinclozolin also binds to recombinant fathead minnow androgen receptor (Wilson, Cardon et al. 2004) and has been found to interfere with mating behavior in male frogs (Hoffmann and Kloas 2010).

IV. Substances screened in ToxCast™ assays

Of the proposed chemicals slated for Phase II EDSP Tier 1 testing, 30 (acetochlor, alachlor, bensulide, clofentzine, clomazone, coumaphos, cyanamide, cyromazine, dicrotophos, diuron,
ethylene thiourea, fenamiphos, fenarimol, fenoxycarb, flumetsulam, hexythiazox, isoxaben, lactofen, lindane, methoxychlor, molinate, oxyfluorfen, paclobutrazol, perfluorocane sulfonic acid, perfluorooctanoic acid, picloram, profenofos, propetamophos, quinclorac, thiophanate-methyl, triflumizole, vinclozolin) have been screened in ToxCast for many endpoints, including endocrine activity. ToxCast is a rapid screening tool that can be used to prioritize substances based on in vitro data combined with existing in vivo, biological pathway and exposure information (Reif, Martin et al. 2010). It has been used to evaluate chemical activities and effects across a broad spectrum of chemical classes (Reif, Martin et al. 2010; Judson, Houck et al. 2010a). ToxCast has been successfully used to screen for potential endocrine activity in oil dispersants used in the Gulf of Mexico following the Deepwater Horizon oil spill in 2010. Because of the urgent need for toxicity data on essentially uncharacterized commercial oil dispersants, a rapid assessment was necessary and highly effective for determining the endocrine disrupting potential of these substances. Judson et al concluded that “…we were able to detect specific bioactivities in complex chemical mixtures for time-sensitive environmental issues and using high-throughput screening assays” (Judson, Martin et al. 2010b). During the October, 2010 NTP Board of Scientific Counselors meeting, Director Birnbaum noted that the methods employed in Tox21 (which includes ToxCast™) offer a “huge opportunity” to screen a large number of environmentally relevant mixtures, with the information gained being used to reduce the number of animal studies required.

In the above mentioned analysis of ToxCast focusing on endocrine activity, methoxychlor and its more active metabolite, HPTE, scored among the highest of the 309 chemicals tested (Reif, Martin et al. 2010). Bensulfide and coumaphos scored relatively high; in contrast, flumetsulam and picloram were predicted to have little to no endocrine activity. Dicrotophos was predicted to be very active in reproductive and developmental assays. This information must be applied to prioritize chemicals on the second list and considered as OSRI for these chemicals.

V. Industrial chemicals

For many industrial chemicals, especially those that have been manufactured for decades and/or to which humans have a history of exposure (e.g. widely used organic solvents or manufacturing intermediates), a wide range of toxicological information exists that should be considered prior to any further testing for these chemicals. Here we present five examples.

A. Acrylamide

“The genotoxic, mutagenic and carcinogenic potentials of acrylamide have been studied extensively,” and several mechanisms of these toxicities have been described and NOAELs established (Erkekoglu and Baydar 2010). Biotransformation of acrylamide by CYP2E1 to glycidamide accounts for a significant proportion of its toxicity, particularly with regard to its “potent” neurotoxicological effects as evaluated in experimentally dosed rats and mice by measuring hind- and forelimb grip strength (Ghanayem, Bai et al. 2010). Metabolism of acrylamide to glycidamide is also required for exposure-induced mutagenicity (Ghanayem, Bai et al. 2010). Consequently, obesity-induced upregulation of CYP2E1 expression exacerbates the reproductive toxicity resulting from acrylamide exposure (Ghanayem, Bai et al. 2010).
Current hypotheses on mechanisms of action for acrylamide’s neurotoxicity suggest that the compound inhibits kinesin-based fast axonal transport and/or directly inhibits neurotransmission (LoPachin, Jones et al. 2003; LoPachin 2004). While its glycidamide-mediated neurotoxicity may account for some of acrylamide’s observed reproductive impacts (which predominantly affect males), multigenerational studies in rats have identified additional mechanisms that contribute to the chemical’s cumulative toxicity profile. Acrylamide increases Leydig cell death and perturbs gene expression levels related to testicular function, including genes involved in apoptosis, cell cycle control, and nucleic acid binding in rats administered 60 mg/kg/day, contributing to sperm defects and abnormal histopathological testicular lesions along with a dose-dependent decrease in serum testosterone (Yang, Lee et al. 2005). Regarding the frequent appearance of thyroid tumors in rats chronically administered acrylamide, acute in vivo studies found a slight increase in serum T4 and some decrease in TSH, “which could suggest a direct stimulation of the thyroid rather than an indirect effect on thyroid regulators” (Khan, Davis et al. 1999; Chico Galdo, Massart et al. 2006). In vitro, acrylamide impacts DNA integrity but shows little influence on “the specific biochemical growth and functional variables of thyroid cells in vitro” (Chico Galdo, Massart et al. 2006).

From these observations, a neuropathy NOAEL has been established at 0.5 mg/kg/d and a fertility NOAEL at 2 mg/kg/d, the latter of which is 2,000-fold greater than the estimated dietary exposure (WHO 2002; Dybing and Sanner 2003; Konings, Baars et al. 2003; Erkekoglu and Baydar 2010). Clearly defined mechanisms of toxicity have been determined for acrylamide and NOAELs have been established for the most potent of those toxicities; again, further testing in EDSP assays would add no regulatory value and is not warranted and acrylamide should be removed from the second list of chemicals for testing.

B. Benzene

“The primary target for adverse systemic effects of benzene following chronic exposure is the hematological system,” and additionally the chemical has been classified by the United States Department of Health and Human Services, the Environmental Protection Agency (EPA) and the International Agency for Research on Cancer as a confirmed human carcinogen based on sufficient data from epidemiological studies and animal evidence (ATSDR 2007).

While essentially all known metabolites of benzene have been shown to decrease erythropoiesis, numerous mechanistic studies have established the central role played by the rapid metabolism of benzene by CYP2E1 (Snyder and Hedli 1996; Smith 1996a; Smith 1996b). CYP2E1 oxidizes benzene to benzene oxide before rearranging to form phenol, benzene’s major initial metabolic product (Vogel and Günther 1967; Jerina, Daly et al. 1968; Lindstrom, Yeowell-O’Connell et al. 1997; Ross 2000). CYP2E1 subsequently oxidizes phenol to catechol or hydroquinone, compounds that are further oxidized by myeloperoxidase (MPO) to 1,2- and 1,4-benzoquinone and other highly reactive quinines that stimulate production of reactive oxygen species and culminate in protein and DNA damage in stem or early progenitor cells (Smith 1996a; Smith 1996b; Nebert, Roe et al. 2002). This damage manifests as hematopoietic and leukemogenic effects (ATSDR 2007). Inhibition of benzene’s metabolism consequently reduces benzene’s toxicity (Andrews, Lee et al. 1977).
Epidemiological case studies consistently “provide clear evidence of a causal relationship between occupational exposure to benzene and benzene-containing solvents and the occurrence of acute nonlymphocytic leukemia, particularly the myeloid cell type (acute myelogenous leukemia)” (Rinsky, Hornung et al. 2002; ATSDR 2007; Integrated Risk Information System (IRIS) 2007). Based on human leukemia data, EPA derived a range of inhalation unit risk values of $2.2 \times 10^{-6} - 7.8 \times 10^{-6} \, (\mu g/m^3)^{-1}$ for benzene (Integrated Risk Information System (IRIS) 2007); for risks ranging from $1 \times 10^{-6}$ to $1 \times 10^{-7}$, the corresponding air concentrations for lifetime exposure range from $13.0 - 45.0 \, \mu g/m^3 \,(4-14 \, ppb)$ to $0.013 - 0.045 \, \mu g/m^3 \,(0.004 - 0.014 \, ppb)$, respectively (Rinsky, Young et al. 1981; Rinsky, Smith et al. 1987). ATSDR notes that benzene is typically in drinking water at concentrations less than $0.1 \, ppb$ and it is typically at the lower end of the observed range of $0.02 - 34 \, ppb$ found in ambient air samples (ATSDR 2007).

As a confirmed human carcinogen, there can be no added regulatory value in determining whether benzene also has endocrine disrupting potential and further testing in the EDSP assays is not warranted and benzene should be removed from the second list of chemicals to be tested.

C. Nitrobenzene

Many of the toxic effects of nitrobenzene (NB) are dependent on enzymatic activation by intestinal flora (following oral exposure); similar activation can be accomplished by various redox reactions in erythrocyte or hepatic microsomes (Levin and Dent 1982; Rickert 1987; Bryant and DeLuca 1991). Principal metabolites include p-nitrophenol, p-nitroaniline and p-aminophenol, with the remaining chemical oxidized to additional nitrophenols or reduced to aniline and other aminophenols (Wang, Zhang et al. 2010a). In this regard, NB shares structure-activity relationships with other aromatic nitro- and amino- compounds that produce common reactive nitroxide intermediates that relate to shared mutagenic and metabolic impacts (Verna, Whysner et al. 1996). These reactive intermediates include the superoxide anion and hydrogen peroxide, which may disturb the redox balance of target cells leading to oxidative stress and ultimately account for many of NB’s toxicological effects (Gutteridge 1995).

Acute, subacute and chronic exposure to NB consistently leads to damage to blood products, the spleen, liver and testes (Bond, Chism et al. 1981; Burns, Bradley et al. 1994; Mitsumori, Kodama et al. 1994; Shimo, Onodera et al. 1994; WHO 2003b; Wang, Zhang et al. 2010a; Wang, Zhang et al. 2010b). Exposure in humans and other animals over a wide range of concentrations leads to the immediate development of methemoglobinemia, a pathological overabundance of oxidized hemoglobin (Goldstein and al 1983; WHO 1986; Maples, Eyer et al. 1990). Abundant evidence suggests that NB’s reactive metabolites accelerate oxidation of hemoglobin to methemoglobin, causing the metalloprotein to lose its ability to reversibly bind oxygen (Smith 1996c; Percy, McFerran et al. 2005; EPA 2009c).

At relatively low subacute doses (30 mg/kg/d in mice exposed for two weeks), a marginal dose-related increase in alanine- and aspartate aminotransferase suggests liver toxicity (Burns, Bradley et al. 1994). In rats, acute administration of relatively high doses of NB has led to the development of hepatocellular nucleolar enlargement at 110 mg/kg, centrilobar hepatocytic
necrosis at 200 mg/kg, and decreasing numbers of spermatozoa in the epididymis accompanied by necrotic debris at 300 mg/kg (Bond, Chism et al. 1981). At 125 mg/kg/day for 28 days in rats, liver, spleen and kidney weights increase while testicular weights decrease with concurrent degeneration of seminiferous tubule epithelium, although these effects tended to abate after a two-week recovery period (Shimo, Onodera et al. 1994). Significant dose-related increases in the incidence of both sperm abnormality and positive micronucleus tests have been observed at approximately 100 mg/kg, or 1/5 LD$_{50}$ (Wang, Zhang et al. 2010a).

Although no human epidemiological data have been analyzed for evidence of potential carcinogenic impacts, two-year inhalational studies exposing mice and rats to up to 50ppm NB have noted an increased incidence of multiple dispersed tumor types which may be related to NB’s oxidation and reduction mechanisms (Maples, Eyer et al. 1990; Cattley, Everitt et al. 1994; Dreher and Junod 1996; Holder 1999). “The distribution of carcinogenicity involves four singular organ sites—lung, mammary gland, kidney, and endometrium: two concordant thyroid responses in male B6C3F1 mice and male F344/N rats, and two concordant liver responses in male F344/N and CD rats. These distributed organ responses present a spectrum of mixed malignancy, species/strain, and sex responses. Altogether, the evidence is 'sufficient' for the determination of NB carcinogenicity in animals (two species, three strains, eight sites) by the inhalation route” (Holder 1999). It is expected that activated NB would bind DNA and protein like other carcinogenic nitroarenes, although either this binding is insufficient for carcinogenesis in all organs or the binding was metabolically reduced at these sites (Holder 1999). “Anticarcinogenic mechanisms in these organs remain unknown, but it is plausible that specific antioxidants were either already present or induced, quenched the nitroxides, prevented binding, and thus protected these tissues” (Netke and al. 1997; Primiano, Sutter et al. 1997; Holder 1999).

**Nitrobenzene has demonstrated hemato-, nephro- and hepatotoxicity and is a rodent carcinogen with described mechanisms of action; there can be little added regulatory value in determining whether nitrobenzene also has endocrine disrupting potential and further testing in EDSP assays is not warranted and nitrobenzene should be removed from the second list of chemicals to be tested.**

**D. Trichloroethylene**

In humans and in experimental animals, trichloroethylene (TCE) exposure leads to an extensively documented array of cancer and non-cancer effects including biochemical, cellular and target organ alterations. TCE toxicity is most evident in the kidneys, liver and nervous system, although the greatest concordance between animal and human data suggests that nephrotoxicity is of greatest concern as most other effects are rarely observed in multiple species (Barton and Clewell 2000; Wartenberg, Reyner et al. 2000; National Research Council (NRC) 2006). Lifetime TCE exposure studies of high doses (GE 500 mg/kg/d for rats and GE 1,000 mg/kg/d in mice) consistently report non-cancer kidney toxicity in both species (National Cancer Institute (NCI) 1976; National Toxicology Program (NTP) 1983; NTP 1988).

Metabolic activation of TCE can occur through a variety of pathways, most importantly via cytochrome P450 oxidation and glutathione conjugation (Pahler, Parker et al. 1999; Lash, Fisher et al. 2000a; Lash, Parker et al. 2000b). The latter pathway is implicated in the generation of
reactive metabolites, S-(1,2-dichlorovinyl)-glutathione (DCVG) and S-(1,2-dichlorovinyl)-L-cysteine (DCVC), associated with various cytotoxic and carcinogenic responses (Lash, Parker et al. 2000b). DCVC has been specifically described as a nephrotoxin (Lash, Parker et al. 2000b).

No reproductive effects have been documented from epidemiologic studies of human populations exposed to TCE, and two-generation studies in mice and rats have generally been negative for a wide range of reproductive endpoints except at very high doses of approximately 1,000 mg/kg/d (Manson, Murphy et al. 1984; Zenick, Blackburn et al. 1984; Cosby and Dukelow 1992; Dawson, Johnson et al. 1993; Agency for Toxic Substances and Disease Registry (ATSDR) 2000). The ability of rat oocytes to be fertilized decreases after exposure to 0.66 g TCE/kg/day for up to 5 days and may be related to observed alterations in oocyte plasma membrane composition, although these changes are not accompanied by any modifications in ovarian gene expression (Berger and Horner 2003; Wu and Berger 2007; Wu and Berger 2008).

A lack of reproductive effects in humans and animals suggests low or no endocrine disrupting potential of trichloroethylene. Other toxicities are well documented however, including neuro, nephro- and hepatotoxicity, and further testing in EDSP assays is not warranted and TCE should be removed from the second list of chemicals to be tested.

**E. Nitroglycerin (glyceryl trinitrate)**

Nitroglycerin (glyceryl trinitrate, GTN), like other organic nitrates, has been extensively studied clinically due to its wide application in the chemotherapeutic treatment of cardiovascular disease. In addition to human data from more than 150 years of clinical use, data from experimental exposure in several species includes information on many of the chronic, developmental and reproductive endpoints sought in EDSP Tier 1 in vivo tests. Most animal toxicity studies on GTN are not readily available in the open literature but are reviewed in a EPA Health Advisory, from which the following information is taken unless otherwise indicated (EPA 1987; Bingham, Cohrssen et al. 2001).

Standard therapeutic doses in humans range between 0.2 to 10 mg/day (Einert, Adams et al. 1963). Requiring only minutes to exert therapeutic effects, GTN is rapidly metabolized to several dinitrate compounds in a variety of tissues and organs, especially in the liver by multiple cytochrome P450 and glutathione S-transferases and elsewhere by mitochondrial aldehyde dehydrogenase (Needleman, Blehm et al. 1971; Bustard, Ryan et al. 2003; Badejo, Hodnette et al. 2010). In the process, nitric oxide (NO) is produced and activates soluble guanylate cyclase to exert GTN’s vasodilatory effects (Bustard, Ryan et al. 2003; Badejo, Hodnette et al. 2010). The principle dinitrate metabolites, glyceryl-1,2-dinitrate and glyceryl-1,3-dinitrate, have a half-life of approximately 15 minutes in humans.

GTN is not highly toxic following acute exposure, with oral LD₅₀s for male rats and mice of 822 and 1,188 mg/kg, respectively. At these doses, indication of toxicity is marked by development of methemoglobinemia and ataxia progressing to death within one to six hours of exposure. In long-term studies, chronic exposure at lower GTP concentrations causes qualitatively and quantitatively different effects among rats, mice, dogs and other mammalian species. Ninety-day studies in rats showed only body weight effects up to 234 mg/kg/day. Anemia and testicular
degeneration are also seen at higher doses up to 1,416 mg/kg. Two-year feeding studies were conducted in both rats and mice (Ellis, Hong et al. 1984). When treated for one year at doses up to 25 mg/kg/day, no effects were observed in rats beyond transient methemoglobinemia (Ellis, Hong et al. 1984). Transient damage to tissues and organs in male NZW rabbits following 26 week dermal GTN exposures of up to 240 mg/kg/day included an increase in kidney and heart weights in the highest dose group. These effects had resolved within five weeks of the final administration (Imoto, Kuramoto et al. 1986a). Rats fed between 363 and 434 mg/kg/day showed decreased body weight gain, methemoglobinemia, and associated effects on erythropoiesis, liver pathology, including cholangiofibrosis, and hepatocellular carcinoma and interstitial cell tumors of the testes. At dietary doses of 31.5 and 38.1 mg/kg/day, the only phenotype observed was a lower incidence of liver lesions than that seen at the higher doses, and no effects were observed at doses about an order of magnitude lower. Mice were less sensitive to chronic GTN exposure, with the high dose of approximately 1000 mg/kg/day leading only to body weight changes and methemoglobinemia with its associated hematologic effects (Ellis, Hong et al. 1984).

Although GTN has not been examined for its capacity to bind estrogen, androgen or thyroid hormone receptors in in vitro assays, reproductive and developmental effects have been observed only following chronic experimental exposure to concentrations significantly greater than that needed to induce a therapeutic effect. A three-generation study in rats exposed to between 408 and 452 mg/kg/day for six months before F0 matings and throughout F1 and F2 generations found a systematic decrease in food consumption and body weight gain, but no clear indication of more extensive impacts in the F0 cohort. Infertility seen in F1 and F2 cohorts was attributed to an increase in interstitial cell tissue and consequently decreased spermatogenesis in males (NovaDel Pharma Inc. 2004). An additional three-generation reproductive toxicity study in rats showed no effects on fertility, viability, growth, or development of offspring at doses up to 38 mg/kg/day. Adverse fertility effects were seen at doses above 363 mg/kg/day which were secondary to malnutrition and testicular tumors. Developmental toxicity studies have been conducted in rats and rabbits given injection doses of up to 20 and 4 mg/kg/day, respectively, during typical gestation administration periods. No teratogenic, embryo toxic, or fetotoxic effects were observed. Aside from transient erythema at site of dermal application, pregnant NZW rabbits administered up to 240 mg/kg/day during days 6 through 18 of gestation showed no signs of decreased or abnormal reproductive performance or fetal development (Imoto, Nakao et al. 1986b).

GTN has produced mixed results in the Ames test but was not mutagenic in a Chinese hamster ovary cell assay without metabolic activation. Chromosomal aberrations were not found in dogs given up to 5 mg/kg/day for nine weeks or in rats given up to 234 mg/kg/day for eight weeks. A dominant lethal test in rats given 3, 32, or 363 mg/kg/day in the diet for 13 weeks also showed no effect on male fertility and no genotoxic activity.

In humans, fatalities from industrial intoxication are uncommon. Medical studies of explosives workers with combined GTN have not found evidence of chronic intoxication or injury despite transient symptoms. An extensive epidemiologic study of 276 factory employees with long exposure to GTN and a related compound, ethylene glycol dinitrate, gave no evidence of permanent deterioration in health (Bingham, Cohrssen et al. 2001). Preliminary examination of data from a randomized, double-blind, placebo-controlled study of early childhood development
of children exposed in utero to GTN found no impact (Guo, Xie et al. 2010).

Summary and conclusions: Extensive studies, including studies in humans, have demonstrated that developmental and reproductive effects are not among the major toxicities of GTN, with the possible exception of testicular degeneration and interstitial cell tumors of the testes. If necessary, information regarding whether GTN is capable of interacting with the EAT pathways could be obtained by estrogen, androgen or thyroid hormone receptor binding studies or other in vitro assessment of GTN’s possible impact on these systems. Additional in vivo tests will be unlikely to provide data that haven’t already been gathered from existing toxicological, pharmacological and epidemiological sources.

VI. Overall Summary and Conclusions

Consideration of exposure is a fundamental element of prioritization of chemicals for risk assessment. In the creation of the second list of chemicals for screening in the EDSP, EPA attempts to consider exposure by focusing not only on pesticides but also on chemicals that may be found in drinking. However, in order to reduce redundant testing, further considerations are necessary, including a first step of evaluating existing toxicological and exposure information for each chemical to determine whether any further testing is warranted, and to streamline any additional testing.

Abundant information exists for many, if not most, of the chemicals on the second list. For many of these chemicals (especially pesticides and industrial chemicals no longer manufactured or imported into the US) it is questionable whether “a substantial population may be exposed…” – an important consideration for prioritization, and also an essential stipulation that allows EPA to request information under SDWA. In addition, there is abundant existing toxicological information for many of these chemicals that renders further testing for endocrine effects moot, either by addressing endocrine-related activity or by characterizing the prevalent toxicity of a given chemical. We have provided such arguments for several chemicals that would warrant their removal from the second list.

A confounding factor in assessing the “added value” of EDSP screening is that it is yet unclear how EPA intends to use the information. For many of the Phase I chemicals, EPA has rejected the equivalent of Tier 2 information since those tests do not address mechanism of action, yet is unclear why such information is necessary. There is currently no requirement or explanation regarding EPA’s intent to regulate a chemical based on an endocrine mechanism of action, particularly when information is available regarding developmental and reproductive effects. Moreover, there is no rationale for requiring information on the possible endocrine activity of chemicals whose major toxicity has already been well characterized (e.g. the neurotoxic activity of acrylamide or the carcinogenic activity of benzene), especially substances banned or severely restricted due to their extreme hazards. This includes other listed chemicals, such as benzo(a)pyrene which although not covered in these comments, also fall into this category. In such cases, any testing to ascertain the likelihood of endocrine activity or mechanism of action is extremely unlikely to affect the chemical’s over-all risk analysis and is therefore a waste of resources and animals. With testing of the second list of chemicals having the potential to kill approximately 70,000 animals, any and all steps toward streamlining the testing must be taken.
Thank you for considering our comments and we look forward to the agency’s positive response.

Sincerely,

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References


Agency for Toxic Substances and Disease Registry (ATSDR) (2000). Toxicological Profile for Polychlorinated Biphenyls (PCBs). (Division of Toxicology/Toxicology Information Branch, US Department of Health and Human Services):  


EPA (2000b). 1,2-Dibromo-3-Chloropropane (DBCP) Hazard Summary.


Rhodes, J. E. and M. Muckerman (1984). Early life-stage toxicity of alachlor to rainbow trout (Oncorhynhus mykiss) under flow-through conditions. Monsanto report number AB-94-280 Monsanto IIA, 8.2.2.1/02.


