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A predictive data-driven framework for endocrine prioritization: a triazole fungicide case study

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ABSTRACT

The US Environmental Protection Agency Endocrine Disruptor Screening Program (EDSP) is a tiered screening approach to determine the potential for a chemical to interact with estrogen, androgen, or thyroid hormone systems and/or perturb steroidogenesis. Use of high-throughput screening (HTS) to predict hazard and exposure is shifting the EDSP approach to (1) prioritization of chemicals for further screening; and (2) targeted use of EDSP Tier 1 assays to inform specific data needs. In this work, toxicology data for three triazole fungicides (triadimefon, propiconazole, and myclobutanil) were evaluated, including HTS results, EDSP Tier 1 screening (and other scientifically relevant information), and EPA guideline mammalian toxicology study data. The endocrine-related bioactivity predictions from HTS and information that satisfied the EDSP Tier 1 requirements were qualitatively concordant. Current limitations in the available HTS battery for thyroid and steroidogenesis pathways were mitigated by inclusion of guideline toxicology studies in this analysis. Similar margins (3–5 orders of magnitude) were observed between HTS-predicted human bioactivity and exposure values and between *in vivo* mammalian bioactivity and EPA chronic human exposure estimates for these products' registered uses. Combined HTS hazard and human exposure predictions suggest low priority for higher-tiered endocrine testing of these triazoles. Comparison with the mammalian toxicology database indicated that this HTS-based prioritization would have been protective for any potential *in vivo* effects that form the basis of current risk assessment for these chemicals. This example demonstrates an effective, human health protective roadmap for EDSP evaluation of pesticide active ingredients via prioritization using HTS and guideline toxicology information.

Abbreviations: abs: absolute; admin: administration; AMA: amphibian metamorphosis assay; A: androgenic pathway; BW: body weight; BWG: body weight gain; d: day; ↓: decreased; dep: dependent; dev: development or developmental; E: estrogenic pathway; ♀: female; GD: gestational day; histopath: histopathology; ↑: increased; inhib: inhibition; ♂: male; mkd: mg per kg BW per day; PND: postnatal day; rel: relative; repro: reproductive or reproduction; S: steroidogenesis; T: thyroid/thyroid pathway; VTG: vitellogenin; wk: weeks; wt: weight

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Supplemental data for this article can be accessed here.

Introduction

The US Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program (EDSP) addresses the statutory requirements of the 1996 amendments to the Safe Drinking Water Act and Food Quality Protection Act. In 2009, EDSP Tier 1 battery test orders were issued, and 50 pesticide active ingredients (PAIs) and two inert ingredients were then evaluated with the EDSP Tier 1 screening assay battery. The EDSP Tier 1 battery includes five *in vitro* assays to assess estrogen, androgen and steroidogenic function and six *in vivo* assays to assess estrogen, androgen, steroidogenic and thyroid function (USEPA 2014a). Conducting all 11 assays requires a minimum of 520 animals and $800,000 USD per chemical (Willett et al. 2011; USEPA 2013). Approximately 10,000 chemicals may be subject to EDSP evaluation (USEPA 2014c), indicating the necessity to develop practical methods to prioritize both PAIs that undergo extensive pre-registration testing and other chemicals (not chemicals with food uses) that constitute most of this list and often lack extensive pre-registration testing. Combined utilization of high-throughput screening (HTS) for bioactivity and exposure may provide the needed efficiency for prioritization and screening.

In the case of endocrine-related adverse outcome pathways (AOPs), HTS bioactivity data are available from EPA's Toxicity ForeCaster (ToxCast™) and Toxicology in the twenty-first century (Tox21) to inform the likelihood that a chemical activates molecular initiating events, e.g., receptor-based interactions, or early key events including upregulation of markers of Phase II metabolism that occur upstream of potential adverse outcomes related to endocrine function. Available (November 2014 release) (USEPA 2014e) HTS assays in Tox21 and ToxCast Phase II specifically related to endocrine outcomes include estrogen receptor (ER), androgen receptor (AR) and thyroid hormone receptor (TR) transcriptional activation assays; estrogen and androgen cofactor recruitment and dimerization assays; ER and AR binding assays; aromatase inhibition assays; and an estrogen-dependent cell proliferation assay. HTS assays indicative of nuclear receptor-mediated hepatic catabolism of thyroid hormones, i.e., nuclear receptor activation assays (ToxCast Phases I and II) and the CellzDirect mRNA expression assays in hepatocytes (ToxCast Phase I only) (Rotroff et al. 2010), may provide some mechanistic information to explain *in vivo* thyroid effects in rodents (Murk et al. 2013; Paul et al. 2014; Sueyoshi et al. 2014; Schraplau et al. 2015). EPA's ToxCast and Exposure Forecasting (ExpoCast) programs (Wambaugh et al. 2014), along with the interagency Tox21 agreement, have yielded a large set of data that may be useful as screening-level information for prioritization tasks. Data from *in vitro* HTS assays may demonstrate a lack of effect on an endpoint or an effect on an endpoint that should be studied in a model of greater biological complexity. Two objectives of the current work are to help define the use of these HTS assay data as first-tier screening information and to highlight the guideline toxicology testing – considered higher-tier information – that might support or refute HTS assay results during EDSP evaluation.

The combined use of physicochemical properties, HTS assay data, exposure predictions and computational tools to characterize the potential for chemicals to trigger molecular-initiating events of AOPs related to endocrine function represents a significant advance in toxicology and risk assessment practice (USEPA 2011, 2013; Browne et al. 2015). In an initial effort to prioritize chemicals for further endocrine activity screening under the EDSP, EPA suggested a tiered consideration of chemical use type, physicochemical properties (acid-dissociation constant, corrosivity and hydrolysis half-life), available quantitative structure relationships, and assay results for endocrine-related activity (USEPA 2012). A classification model developed for endocrine activity using ToxCast and Tox21 HTS assay data previously demonstrated that available HTS data correctly indicated estrogenic/anti-estrogenic and androgenic/anti-androgenic activity in the relevant corresponding Tier 1 assays (Rotroff et al. 2013). Further, results from a predictive ER activity model corresponded qualitatively to the results of estrogen-related Tier 1 assays, including the ER binding and transcriptional activity assays and the uterotrophic assay (Rotroff et al. 2014; USEPA 2014b; Browne et al. 2015). Thus, hazard screening information from the predictive ER activity model (Rotroff et al. 2014; USEPA 2014b; Judson et al. 2015; Browne et al. 2015) and the predictive AR model under development (USEPA 2014b) are useful in determining a lack of ER or AR pathway-based hazards, indicating no need for the corresponding Tier 1 assays. Conversely, these ER and AR activity models may demonstrate a potential *in vitro* estrogen or androgen activity for a chemical that could be further evaluated using estrogen- or androgen-sensitive *in vitro* and *in vivo* assays. The continuing development of models that integrate results from multiple assay technologies and data sources in a pathway-based approach will ultimately decrease the amount of animal-based EDSP Tier 1 screening required.

To assess whether the use of HTS-based prioritization would be human health protective when compared with current risk assessment practices, a case study of three data-rich triazole fungicides – myclobutanil, propiconazole and triadimefon – was developed. These triazole fungicides were selected because they have been studied in a variety of models, with data available not only from EDSP Tier 1 screening but also from other scientifically relevant information (OSRI), including guideline toxicology studies,

published studies, ToxCast/Tox21 assays and ExpoCast. Further, although these triazoles share structural features and a common fungicidal mode-of-action (Trosken et al. 2006a), the apical toxicities, including those potentially related to the endocrine system, manifest differently *in vivo* by triazole. Previously, these three triazole fungicides had been selected by the EPA Office of Research and Development (Hester et al. 2006; Tully et al. 2006; Wolf et al. 2006) for extensive *in vivo* and *in vitro* testing to explore their varying effects related to hepatic-, thyroid-, reproductive- and endocrine-related endpoints. We explored whether HTS bioactivity and exposure predictions would have created an initial prioritization for further endocrine activity screening that would be as human health protective as the traditional testing and risk assessments performed as part of the registration of these triazoles. First, a comparison of the three "lines of evidence" – including HTS bioactivity results, the EDSP Tier 1 battery plus published OSRI, and guideline toxicology studies – was assessed. Next, the margin between HTS bioactivity and human exposure predictions was compared with the margin separating mammalian *in vivo* bioactivity and chronic human exposure estimates derived by EPA for the registered uses of these chemicals. A key goal was to illustrate whether utilization of HTS-based prioritization would have been as protective as the point-of-departure currently used in risk assessment. The resultant case study provides a methodology and support for the utilization of HTS information in prioritization applications, a course currently suggested by EPA (USEPA 2015b) and likely to be improved as more HTS methods are developed and refined for this purpose. Based on the evaluation of these three triazoles, four questions are considered:

1. Are HTS assay data currently sufficient for an endocrine prioritization task, and does the current HTS battery (ToxCast/Tox21) provide appropriate biological coverage for each of the sub-types of endocrine pathways (estrogen, androgen, thyroid and steroidogenesis)?
2. Are HTS information, guideline toxicology data and the EDSP Tier 1 assays sufficiently concordant to support that HTS prioritization would be possible? In other words, are there major biological gaps or false negatives in HTS that would have affected EDSP evaluation and risk assessment for these three PAIs? Although false positives in HTS may indicate a need for additional evaluation, higher-tier testing (other *in vitro* or *in vitro* assays) would eventually reveal the false positive, erring on the side of conservatism.
3. Is prioritization based on the high-throughput exposure and bioactivity predictions as protective of human health as traditional risk assessment approaches?
4. In the absence of the EDSP Tier 1 battery, could human health protective prioritization decisions be made regarding the need for any additional hazard and exposure information, using HTS prioritization alone or in combination with the available 40 Code of Federal Regulations (CFR) Part 158 guideline toxicology study information?

Methods

High-throughput screening hazard prediction

The *in vitro* HTS assay data used in this evaluation were generated by the ToxCast and Tox21 research programs and are publicly available (http://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data) (Kavlock et al. 2012; Tice et al. 2013). As of the November 2014 data release, more than 800 assay endpoints were available via ToxCast and Tox21, with more than 400 assay endpoints available for each of the three triazoles in the present evaluation. All three triazole fungicides were in the initial ToxCast Phase I chemical library, and results are available for ToxCast Phase II assays (USEPA 2014e) (invitrodb_v1, accessed November 2014, http://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data), as well as previously released ToxCast Phase I assay data (December 2009, http://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data). A subset of the ToxCast Phase I assay dataset from the CellzDirect assay technology was selected for the current exercise because of its potential utility in predicting chemical induction of hepatic catabolism and transport of thyroid hormones via nuclear receptor activation (Rotroff et al. 2010). This assay technology utilized primary human hepatocytes ($N = 2$ donors) exposed to test chemicals for 6, 24 or 48 h with an assay endpoint of increased mRNA expression of xenobiotic metabolizing and transport enzyme targets, and at the time of this analysis, was included in archived data with only AC_{50} values available. Dose–response information and any dose–response curve-fitting caution flags are now publicly available for all of the data used in this report.

Endocrine-related HTS assay endpoints were selected and assigned to endocrine target hypotheses: estrogenic (E), anti-estrogenic (anti-E), androgenic (A), anti-androgenic (anti-A), steroidogenesis (S), thyroid (T) and anti-thyroid (anti-T). Assignment of the E/anti-E or A/anti-A hypotheses to specific HTS assays was based on description of the computational models developed by EPA for predicting receptor-based endocrine activity (USEPA 2014b; Browne et al. 2015). For the present exercise, assay endpoints related to S/T/anti-T were also considered based on the expert judgment. The numbers of HTS assay endpoints from ToxCast and Tox21 considered as a line of evidence for potential endocrine bioactivity hypotheses are illustrated in Figure 1 and the full assay names and descriptions are listed in Supplemental Table 1.

For additional comparison, scores from the ToxCast ER and AR agonist and antagonist area under the curve (AUC) models, developed by the EPA Office of Research and Development (USEPA 2014b; Browne et al. 2015), were compared with the HTS assay results. These ER and AR AUC models integrate multiple HTS assay results for ER and AR agonist and antagonist activity and use a systems biology approach to consider pathway activity along with potential sources of assay interference, in practice providing more context for interpreting HTS assay results, e.g., reducing the impact of false positives, for ER and AR activity. These model scores are also available in the EDSP21 Dashboard (actor.epa.gov/edsp21). AUC values ranged from 0.0 to 1.0 for the ER and AR pathways, with a greater value indicative of a greater

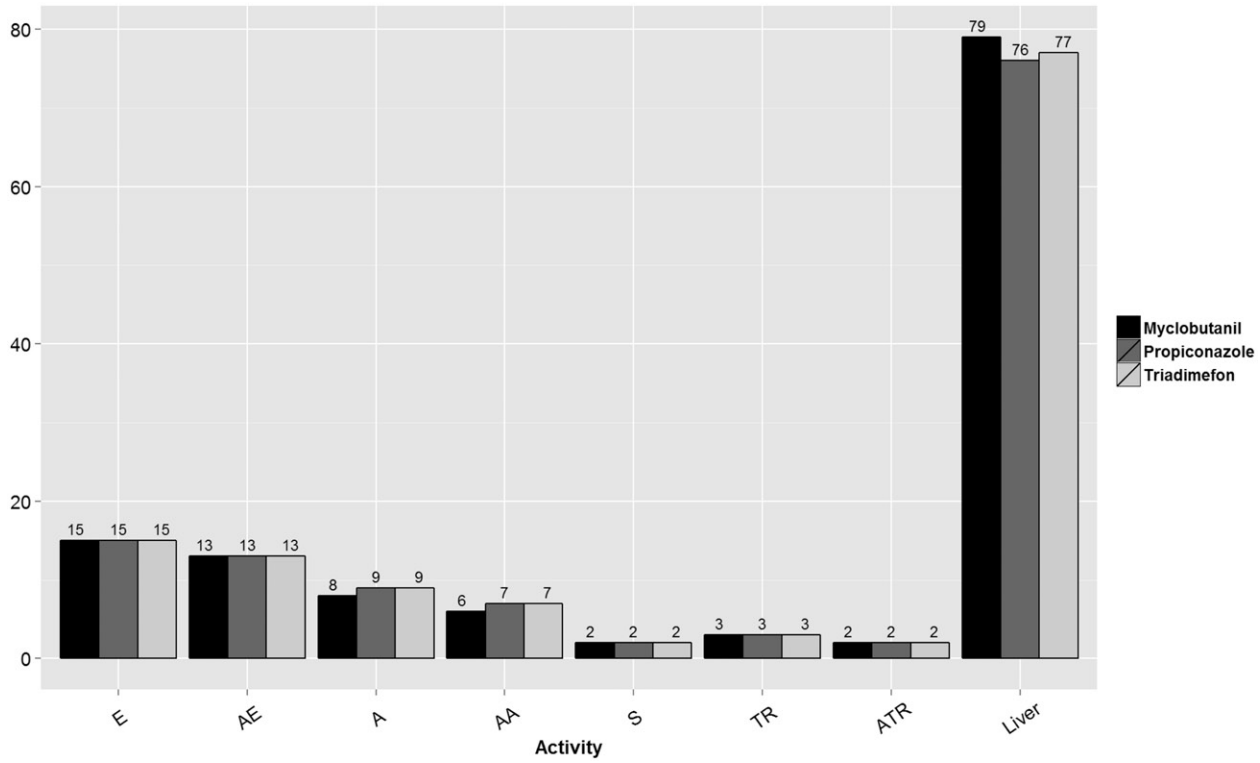

Figure 1. Number of HTS assays available for the three triazoles within each endocrine activity hypothesis. The assay endpoints available by estrogen (E), anti-estrogen (AE), androgen (A), anti-androgen (AA), steroidogenesis (S), thyroid receptor (TR), anti-thyroid receptor (ATR) and hepatic catabolism related to thyroid hormone (Liver). Not all triazoles were tested in all assays, leading to minor discrepancies between triazoles for the number of assay endpoints available.

response. The estrogen, anti-estrogen and androgen model scores must surpass a threshold of 0.1 to be considered positive, and the anti-androgen model score must surpass a threshold of 0.05 to be considered positive (USEPA 2014b).

In vitro *to* in vivo *extrapolation of oral equivalent doses for HTS assay* AC_{50} *values*

In vitro-to-*in vivo* extrapolation of oral equivalent doses for HTS assay concentrations that achieved 50% maximal activity (AC_{50}) facilitates comparison of HTS assay data with oral doses administered in whole animal studies. A simplified derivation of oral equivalent doses was employed that applies only to first-order metabolism; it used the following assumption (Wetmore et al. 2012):

$$\left(\frac{\text{ToxCast AC50 } (\mu\text{M}) \times \left(1\frac{\frac{mg}{kg}}{d}\right)}{Css\ 95\%\ (\mu\text{M})}\right) = \text{Oral equivalent}\left(\frac{\frac{mg}{kg}}{d}\right)$$

Steady-state concentrations (Css) in blood were predicted from measured intrinsic clearance and fraction unbound for each triazole and other standard physiological parameters (Wetmore et al. 2012). The upper 95th percentile of the potential distribution of Css values (Css95%) was estimated in SimCyp using a Monte Carlo approach to simulate population variability (Wetmore et al. 2012; Wetmore 2014). These high-throughput toxicokinetic predictions of the Css95% are publicly available (USEPA 2014b) (Appendix 3). The Css95% values used to calculate the oral equivalent doses for the AC50 values for myclobutanil, propiconazole and triadimefon are listed in Table 1. All the calculated oral equivalent doses for the endocrine-related HTS assay AC50 values in this case study, used in Figures 2–4, are available in Supplemental File 2.

Table 1. Steady state systemic concentration values used to calculate oral equivalent doses.

PAI	Css95%* (μM)
Myclobutanil	0.570242
Propiconazole	1.108062
Triadimefon	0.327346

*Css95% values (human) equivalent to a 1 mkd dose level, from (US EPA, 2014b, Appendix 3).

High-throughput human exposure prediction (ExpoCast)

The ExpoCast project is a rapidly evolving program at EPA intended to predict human exposure to chemicals with limited or no exposure-related data (Wambaugh et al. 2013, 2014). The second-generation analysis employs Bayesian methods to infer exposure ranges that align with National Health and Nutrition Examination Survey (NHANES) data, using chemical descriptors (chemical use type and production volume) and demographics to calibrate the regression coefficients for the predictive model. Note that the purpose of the current ExpoCast model is to provide an approximate range of human exposures for use in prioritization efforts. The current ExpoCast model uses five indicators of exposure, including four use categories (industrial and consumer use, pesticide inert, pesticide active and industrial with no

consumer use) and production volume (expressed in logarithmic scale), as inputs for a model to predict exposure. These five factors accounted for approximately 50% of the variability in exposures measured in NHANES across demographic groups. Demographic groups included in this analysis mirrored those in NHANES: children ages 6–11 and 12–19 years; adults ages 20–65 and greater than 65 years; body mass index greater than 30; females; males; and reproductive-age females (16–49 years). Demographic group parameters did not appear to be significant for predicting different levels of exposure in this version of the model (Wambaugh et al. 2014).

The predicted human oral exposure range for each of the three triazoles, from the median to the 95th percentile, for the total population was selected from the ExpoCast project second-generation analysis for the comparisons in this manuscript (Wambaugh et al. 2014) (Supplemental Table 1); the total population median and total population upper 95% predicted exposures for myclobutanil, propiconazole and triadimefon are listed in Table 2. The total population exposure range was selected for comparison because the ExpoCast predictions ranged two orders of magnitude, and perhaps because of the large uncertainty around the possible range of exposure, the total population-predicted exposure range was roughly equivalent to the ranges predicted for all the various subpopulations in the second-generation ExpoCast analysis.

The ExpoCast project has not yet extended to prediction of ecological exposures for pertinent species or concentrations in environmentally relevant matrices beyond a nascent research stage, though this is the subject of ongoing research (USEPA, 2014c). Further, prediction of relevant ecological exposures is beyond the scope of this manuscript, which is focused on human health applications. Thus, the analysis presented in this paper using HTS bioactivity and exposure predictions is not yet possible for environmental prioritization applications, and, therefore, cannot be evaluated for its utility for ecological risk assessment at this time.

Exposure estimates from EPA human risk assessment documents

To facilitate comparison, a range of the exposure estimates for each PAI is presented in Figures 2–4, using the largely unrefined chronic aggregate exposure values from EPA risk assessment documents. The estimated chronic aggregate (dietary and drinking water) exposure range for all subpopulations and the total US general population, the model used and the reference for these values are provided in Table 3. It should be underscored that these values include both dietary and drinking water exposures and are unrefined estimates of exposure based on modeling data. The standard EPA approach is to conduct initial, conservative first-tier exposure assessments and subsequently refine the model inputs only if the level of concern is exceeded using conservative inputs. These exposure assessment estimates are considered conservative and protective of human health and are not necessarily accurate measurements of exposure. Possible refinements to the estimates derived from dietary exposure models include the percentage of crop treated, i.e., the proportion of the marketplace treated with a particular PAI; the use of US Department of Agriculture Pesticide Data Program residue monitoring data in place of tolerance values; and the use of

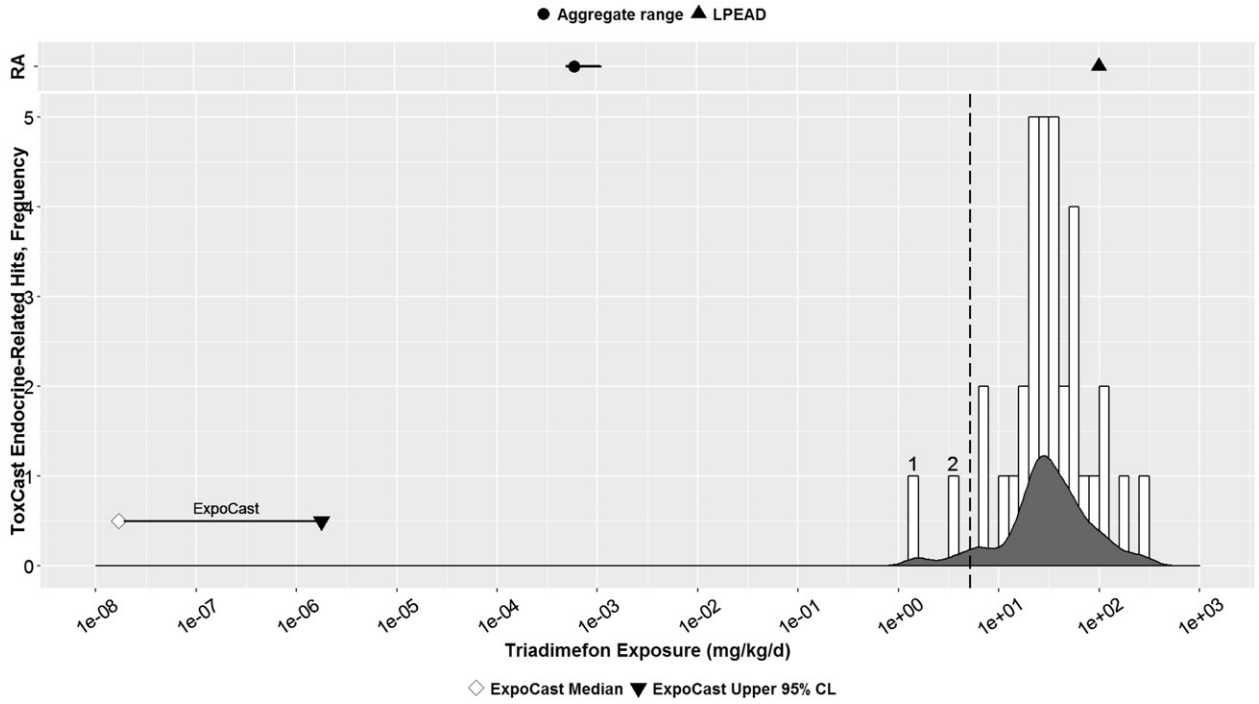

Figure 2. Triadimefon: Parallel comparison of predicted bioactivity and exposure and US EPA chronic exposure estimates and *in vivo* bioactivity. Annotated to show Bins 1–2. See text, Table 6, and Supplemental File 2 for additional details on AC_{50} by assay and bin. Dotted line = cytotoxicity caution flag (1.68 μM or 5.13 mkd). LPEAD, lowest potentially endocrine active dose (= 1800 ppm or 100 mkd for triadimefon).

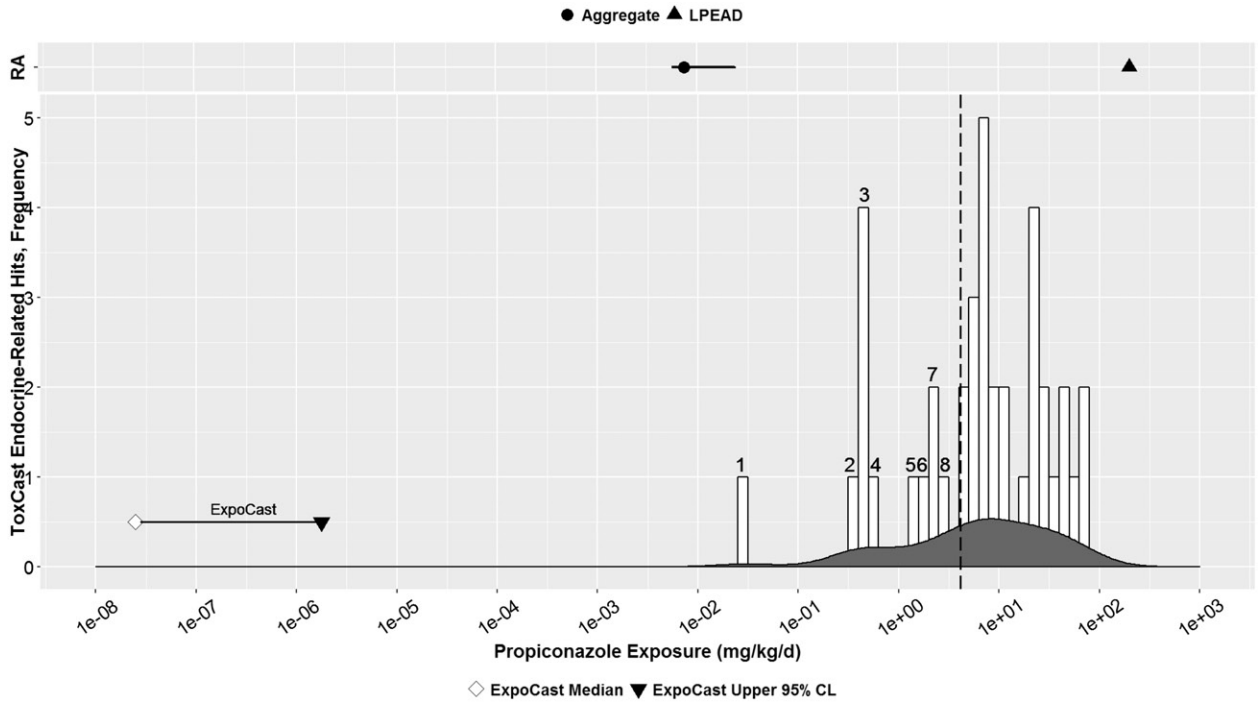

Figure 3. Propiconazole: Parallel comparison of predicted bioactivity and exposure and EPA chronic exposure estimates and *in vivo* bioactivity. Annotated to show Bins 1–8. See text, Table 7, and Supplemental File 2 for additional details on AC_{50} by assay and bin. Dotted line = cytotoxicity caution flag (4.6 μM or 4.15 mkd). LPEAD, lowest potentially endocrine active dose (= 2500 ppm or 200.4 mkd for propiconazole).

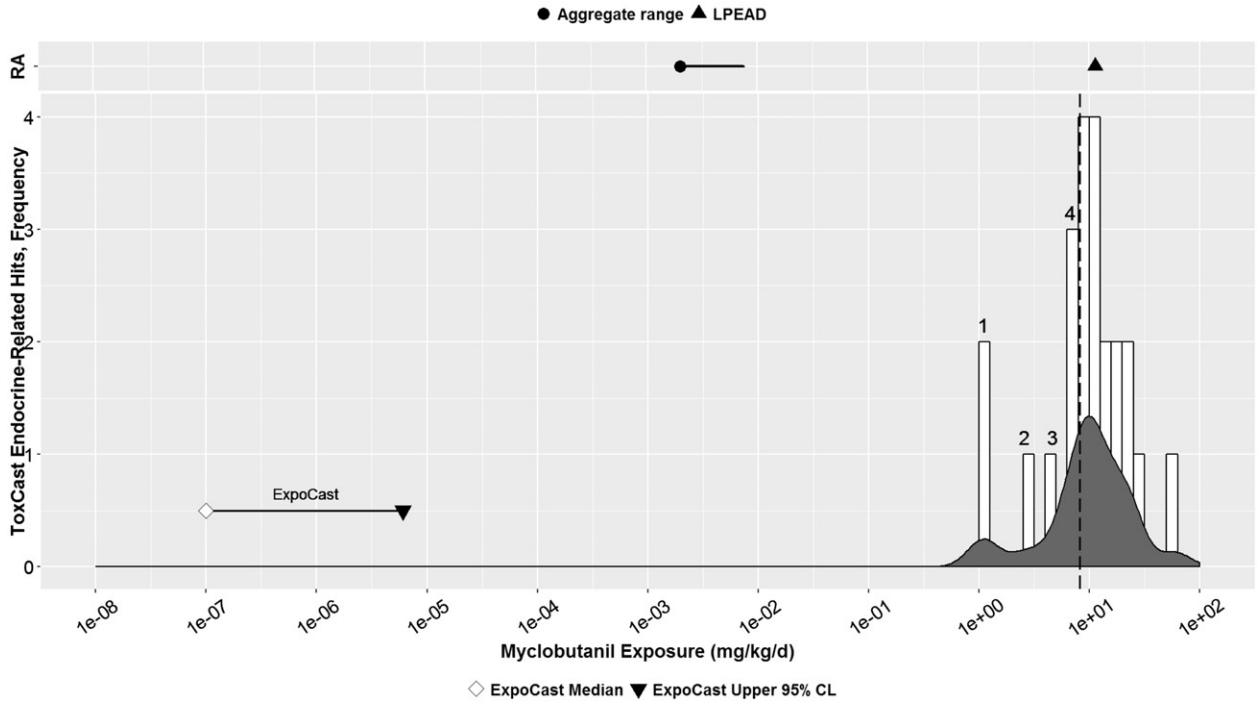

Figure 4. Myclobutanil: parallel comparison of predicted bioactivity and exposure and EPA chronic exposure estimates and *in vivo* bioactivity. Annotated to show Bins 1–4. See text, Table 8, and Supplemental File 2 for additional details on AC_{50} by assay and bin. Dotted line = cytotoxicity caution flag (4.69 μM or 5.13 mkd). LPEAD, lowest potentially endocrine active dose (= 200 ppm or 9.84–12.86 mkd for myclobutanil). For graphing purposes, the midpoint of the interval between 9.84 and 12.86, or 11.35 mkd, was used as the LPEAD.

specific (measured) food processing factors in place of default processing factors. Unrefined estimates of drinking water concentration are one to five orders of magnitude higher, with the majority between three and five orders of magnitude higher, than actual measured values, because a series of conservative assumptions are applied. These assumptions include the following: every acre of the watershed is treated at the maximum single application rate, with the maximum number of applications; all fields in the catchment are assumed to be treated at the same time; and runoff instantaneously moves to the drinking water reservoir (Jones 2005; Winchell & Snyder 2014).

The EDSP Tier 1 battery and published OSRI

The methods and availability of EDSP Tier 1 battery studies (Series 890) and key publications that, in addition to guideline studies, constituted the OSRI used to support EDSP Tier 1 test waivers for myclobutanil, propiconazole and triadimefon are described in Table 4. The EDSP Tier 1 battery consists of 11 different assays that assess the potential for a test compound to perturb the estrogen, androgen or thyroid systems, or interfere with steroidogenesis. Analysis of the Tier 1 screening battery results for each of these endocrine pathways considers the strength of responses, consistency across studies, plus all other supporting data (e.g., OSRI) that can be used to indicate whether or not a particular endocrine mechanism may be perturbed. The EDSP Tier 1 battery is considered to be screening and, therefore, may not demonstrate adversity or a complete dose-response. Studies in non-mammalian species, including a fish short-term reproduction study (FSTRA) and an amphibian metamorphosis assay (AMA), are included in the EDSP Tier 1 battery (methods described in Table 4). Results of these non-mammalian assays are described only briefly in this manuscript (Tables 9b, 10b, and 11b in Supplemental File 4), as these results are relevant to comparisons with ecological exposures that are not considered in this manuscript. For data rich compounds, such as PAIs, however, data are often available in more human-relevant species from 40 CFR Part 158 guideline toxicology studies (both in terms of taxonomy and in terms of exposure) in relation to reproduction and thyroid homeostasis. Therefore, the scope of this manuscript does not extend into detailed descriptions of all ecotoxicology data for these data-rich compounds, and instead focuses on two key objectives: (1) examination of the concordance across the three lines of evidence related to human/mammalian health effects; and (2) facilitation of a revised EDSP framework for data-rich chemicals via consideration of both human exposure estimates and potential endocrine activity. Qualitative review of the studies used to satisfy the EDSP Tier 1 data requirements is provided in Tables 9–11; any potential effects on the endocrine system are reported by pathway (estrogen, anti-estrogen, androgen, anti-androgen, steroidogenesis or anti-thyroid), and any other findings in the study (e.g., decreases in body weight [BW], changes in liver weight, or histopathology) are all reported in a separate column.

Table 2. Predicted human oral exposure range for three triazoles.

PAI	Total population median (mg/kg/d)	Total population upper 95% (mg/kg/d)
Myclobutanil	9.916 E-8	6.022 E-6
Propiconazole	2.476 E-8	1.766 E-6
Triadimefon	1.7 E-8	1.765 E-6

These exposure predictions reflect the total population. From Wambaugh, et al. (2014).

Guideline toxicology studies relevant for demonstrating potential endocrine activity

PAIs are evaluated in a system of required guideline toxicity tests in mammals for registration for use. Together, these guideline studies constitute an important line of evidence for predicting endocrine bioactivity (Table 5). The multigenerational study in rodents provides the most endocrine-relevant information, including observation of reproductive performance, pregnancy and sexual maturation across two generations. Developmental toxicity studies in rodents and rabbits provide an assessment of reproductive and endocrine parameters in dams and offspring following gestational exposure. The developmental neurotoxicity study provides assessment of hypothalamic-pituitary-thyroid axis function following perinatal (gestational and postnatal) exposure. Several other types of studies, including sub-chronic, chronic and carcinogenicity studies, yield relevant information on endocrine tissues following repeat dosing. The presence of an apical effect in a guideline study may suggest more than one endocrine or non-endocrine hypothesis as to its origin. Often a lack of effects on endpoints related to endocrine function is more conclusive than the presence of an effect, especially when that effect may be caused by one or more modes-of-action (MOAs). Qualitative review of these studies is presented in Tables 12–14; again, any effects considered potentially endocrine-relevant are listed by pathway. Any additional effects (e.g., decreases in BW, changes in liver weight, or histopathology) are also listed in Tables 12–14 in a separate column. Key endpoints in 40 CFR Part 158 guideline toxicology studies related to endocrine system function, along with the limitations to their interpretation, are described in Table 5.

Results

HTS information: line of evidence 1

Tables 6–8 present the HTS results for the three triazoles, organized by endocrine hypothesis, similar to the system of endocrine hypotheses suggested by Borgert et al. (2014), including steroidogenesis (S), estrogenic (E), anti-estrogenic (anti-E), androgenic (A), anti-androgenic (anti-A), thyroid

Table 3. Chronic aggregate exposure estimates from EPA risk assessments.

PAI	Range (min–max) across sub-populations (mg/kg bw/d)	Total US general population (mg/kg-bw/d)	Model note	Reference
Myclobutanil	0.002882–0.007569	0.005013	DEEM-FCID ver 2.03 (with refined inputs such as USDA PDP data for three crops and average % crop treated for 25 crops with remainder at 100% crop treated)	USEPA (2007c, 2008)
Propiconazole	0.005620–0.023722	0.007434	None	USEPA (2014d)
Triadimefon	0.000503–0.001108	0.000607	Water model is "Entire Golf Course" (DEEM-FCID). As use is largely restricted to turf-use, chronic exposure is not anticipated	USEPA (2009)

Table 4. EDSP Tier 1 assays plus published OSRI for the three triazoles.

OCSPP guideline	Test name	Method	Myclobutanil	Propiconazole	Triadimefon
890.1100	Amphibian metamorphosis (frog)	21 d continuous flow-through exposure of *Xenopus laevis* tadpoles and measurement of development and markers of thyroid function	Y	Y	N; Goetz et al. (2007), Wolf et al. (2006), and Rockett et al. (2006)
890.1150	Androgen receptor binding (rat prostate)	20 h exposure using rat ventral prostate as a source of cytosolic AR, measuring displacement of R1881	Y	N; Bauer et al. (2002) supported by Kojima et al. (2004)*	Y
890.1200	Aromatase (human recombinant)	15 min exposure using recombinant human aromatase to measure inhibition of enzymatic conversion of ^{3}H-androstenedione	Y	N; Ohno et al. (2004), supported by 4 other studies†	N; USEPA (2007b), Ohno et al. (2004), and Goetz et al. (2009c)
890.1250	Estrogen (receptor Binding (ER-RUC)	16–20 h exposure using rat uterine cytosolic ERα, measuring displacement of 17β-estradiol	Y	Y	Y
890.1300	Estrogen Receptor Transcriptional Activation (HeLa-9903)	20–24 h exposure with measurement of hERα activation as a transcription factor to upregulate luciferase production in stably transfected HeLa cells	Y	N; Kjaerstad et al. (2007); Kojima et al. (2004); Hurst and Sheehan (2003)‡	Y
890.1350	Fish short-term reproduction (FSTRA)	21 d continuous flow-through exposure of sexually-mature fathead minnows followed by measurement of reproductive success	Y	Y	Y
890.1400	Hershberger (rat)	Oral exposure to castrated male CD rats from PND 55-64, followed by measurement of accessory sex tissue weights	Y	N; Goetz et al. (2007), Kjaerstad et al. (2007) supported by Taxvig et al. (2008) and Tully et al. (2006)	Y
890.1450	Female pubertal (rat)	Oral exposure to juvenile female CD rats from PND 22-42 followed by measurement of clinical chemistry, age and BW at VO, tissue weights (pituitary, thyroid, liver, kidneys, adrenals, uterus, ovaries), histology (uterus, ovary and thyroid, kidney), serum T4 and TSH, and estrous cycle	Y	N; Rockett et al. (2006), supported by: Goetz et al. (2007) and Wolf et al. (2006)	N; Rockett et al. (2006)
890.1500	Male pubertal (rat)	Oral exposure to juvenile male CD rats from PND 23-53, followed by measurement of clinical chemistry, age and BW at PPS, tissue weights (pituitary, thyroid, liver, kidneys, adrenals, testes, and accessory sex organs), and histology (epididymis, testis, thyroid, kidney), and serum T4, TSH and testosterone	Y	N; Goetz et al. (2007), supported by: Tully et al. (2006).	N; Goetz et al. (2007); supported by Goetz et al. (2009c), Tully et al. (2006), and Wolf et al. (2006)
890.1550	Steroidogenesis (human – H295R)	48 hr exposure in the H295R human adrenocortical carcinoma cell line followed by measurement of testosterone and estradiol production in the cell culture medium	Y	N; Goetz et al. (2009) and Kjaerstad et al. (2007)	N; Goetz et al. (2009)
890.1600	Uterotrophic (rat)	Oral exposure to immature female CD rats from PND18-21, followed by measurement of uterine weight.	Y	Y	N; Rockett et al. (2006)
Goetz et al. (2007)	Male reproductive developmental landmarks	Dietary exposure from GD6 to PND120. One ♂ per litter was evaluated at PND1, 22, 50 and 92. Measurements included:	Y	Y; supported waivers for the Hershberger and Female and male pubertal assays	Y; supported waivers for the female and male pubertal assays

(*continued*)

Table 4. Continued

OCSPP guideline	Test name	Method	Myclobutanil	Propiconazole	Triadimefon
		• Litter size, gender ratio and wt • AGD at PND0 • BW and abs and rel organ wts (liver, testis, ventral prostate, epididymis and seminal vesicle) • Liver histopath • Serum hormone luteinizing hormone • Serum estradiol, testosterone, T4, T3 and TSH • Age and BW at PPS • Sperm count, morphology and motility • Fertility and fecundity			
Goetz et al. (2009)	Evaluation of steroidogenesis	• H295R steroidogenesis assay with measurement of estradiol, progesterone, and testosterone in the cell culture medium • Adult and neonatal rat *in vitro* testis culture with measurement of testosterone, androstenedione, 17α-hydroxyprogesterone and progesterone production • Dietary 1800 ppm triadimefon exposure from PND60 to PND90 in adult ♂ rats with measurement of: BW; liver, epididymis, ventral prostate, seminal vesicle, testes, pituitary wts; intratesticular testosterone production; serum testosterone	Y; H295R and testis culture only	Y; H295R only	Y; all three study parts; supported waiver of steroidogenesis and male pubertal assays
Hester and Nesnow (2008)	Gene expression analysis in rat thyroid	30 or 90 d dietary exposure in ♂ rats followed by gene array analysis of thyroid tissue	Y	N	Y; supported waiver for amphibian metamorphosis assay
Kjaerstad et al. (2007)	Anti-androgenic Hershberger Assay	Similar to Rat Hershberger Assay (OCSPP 890.1400)	N	Y; supported waivers for ER-TA, Steroidogenesis, and Hershberger Assays	N
Rockett et al. (2006)	Female reproductive developmental landmarks	Dietary exposure from GD6 to PND92. Measurements included: • Litter size, gender ratio and wt • AGD at PND0; • BW and abs and rel organ wts, inc liver, pituitary, hippocampus, hypothalamus, thyroid, ovaries, drained uterus • Liver, ovary, and thyroid histopath • Serum estradiol levels • Age at VO • Estrous cyclicity	Y	Y; supported waiver for the female pubertal assay	Y; supported waivers for the amphibian metamorphosis, female pubertal, and uterotrophic assays
Taxvig et al. (2008)	Short-term *in vivo* endocrine studies	• Study 1: rat Hershberger Assay similar to OCSPP 890.1400 • Study 2: gestational study: pregnant ♀ dosed by gavage GD7-GD20, with Cesarian section on GD21, followed by analysis of post-implantation loss, AGD, and testicular testosterone in foetuses	N	Y; supported waiver for Hershberger Assay	N

(*continued*)

Table 4. Continued

OCSPP guideline	Test name	Method	Myclobutanil	Propiconazole	Triadimefon
Tully et al. (2006)	Testis and hepatic biomarkers	Oral gavage exposure to adult ♂ rats for 14 d followed by measurements including: • BW & rel organ wts, including liver, spleen, adrenal, testis, epididymis, seminal vesicle, ventral prostate and brain • Liver histopath • Serum testosterone, FSH, LH and estradiol • Sperm morphology and motility • Hepatic and testis gene expression analysis	Y	Y; supported waivers for the Hershberger and male pubertal assays	Y; supported waiver for male pubertal assay
USEPA (2007b)	Aromatase inhibition assay	15 min exposure using recombinant human aromatase to measure inhib of enzymatic conversion of ^{3}H-androstenedione. This was the US EPA validation study for this assay	N	N	Y; supported waiver of aromatase inhibition assay
Wolf et al. (2006)	Markers of thyroid disruption	Dietary exposure to adult ♂ rats for 4, 30 or 90 d followed by measurements including: • BW and liver wt • Liver and thyroid histopath • Serum T3, T4, TSH and cholesterol • EROD, PROD, MROD and UDPGT activity in liver microsomes	Y	Y; supported waiver for the female pubertal assay	Y; supported waivers for the amphibian metamorphosis and male pubertal assays

The EDSP Tier 1 battery and relevant publications used as OSRI are described. For each of the three triazoles, the EDSP Tier 1 assay data submitted and published OSRI used to fulfill the EDSP data requirements, are described. (Y = yes, study conducted; N = no, study was not conducted; OSRI used in place of the study are cited).

*These studies are described in more detail in Tables 9–11, and represent similar *in vitro* study designs to assess androgen receptor binding or transactivation.

†These studies are described in greater detail in Tables 9–11, and all represent similar *in vitro* study designs to evaluate aromatase inhibition.

‡Kojima et al. (2004) and Hurst and Sheehan (2003) are described in greater detail in Tables 9–11, and represent similar *in vitro* study designs to evaluate estrogen receptor transactivation.

receptor agonist (TR) and thyroid receptor antagonist activity (anti-TR), as well as hepatic catabolism and thyroid hormone clearance (liver). Some of the HTS assays are repeated if they can be assigned to more than one hypothesis, e.g., an ER binding assay can inform both anti-estrogenic and estrogenic hypotheses. The number of positives out of the total number of assays for each hypothesis and the AC_{50} (μM) for each positive assay endpoint ID are indicated for the relevant endocrine hypothesis. The estimated "cytotoxicity limit" provided in the EPA EDSP21 Dashboard (actor.epa.gov/edsp21, Bioactivity tab) for each chemical is also indicated to assist in interpretation of these data; this cytotoxicity limit is simply a useful prediction, but not necessarily a strict "cut-off" for reporting these assay data. AC_{50} values at concentrations that exceed the cytotoxicity caution limit may represent assay positives of little to no biological relevance, and may have resulted from assay interference rather than selective *in vitro* activity. In addition, the available ToxCast AUC ER and AR model scores are reported for four hypotheses: E, anti-E, A and anti-A (USEPA 2014b; Browne et al. 2015). Pathway models for steroidogenesis and thyroid disruption are not yet available.

The HTS data for triadimefon, propiconazole and myclobutanil can be compared and contrasted as a group based on the results shown in Tables 6, 7 and 8, respectively. In general, the HTS data suggest that these three chemicals activate markers of hepatic metabolism, possibly via interaction with nuclear receptors, including the constitutive androstane receptor (CAR) and pregnane-X receptor (PXR), that are consistent with previously published studies (Tully et al. 2006; Goetz & Dix 2009). HTS data also suggest that all three triazoles inhibit aromatase *in vitro*, but they have differing potency in these *in vitro* assays. Each of the three triazoles shows little to no potential to interact with ER and AR pathways directly. A detailed review of HTS information (from November 2014 release) for these three triazoles is described in Supplemental File 5.

EDSP Tier 1 assays plus published OSRI: line of evidence 2

An overview of the EDSP Tier 1 assays plus published OSRI for each chemical is provided in Tables 9–11. A detailed, narrative review of the EDSP Tier 1 assays plus published OSRI for each chemical is provided in Supplemental File 5.

Effects on endocrine-sensitive reproductive measures were also assessed in the FSTRA (890.1350; see Supplemental File 4). The AMA (890.1100; Supplemental File 4) was used to screen for potential effects on thyroid signaling. This

Table 5. Guideline toxicity studies.

Study type	EPA	OECD	Endpoints of endocrine relevance	Additional considerations
Subchronic rodent Subchronic dog	870.3100 870.3150	408 409	Following repeat dose exposure at 90 d, measurement of weights (liver, kidneys, adrenals, gonads, brain (rat), thyroid (dog), gross pathology and histopathology of numerous tissues, including endocrine-relevant tissues (pituitary, brain, thyroid/parathyroid, adrenals, testis, ovary, uterus, epididymis, prostate, seminal vesicles, and, if indicated by signs of toxicity, mammary gland), in male and female animals	Weights and pathology, while providing valuable information, do not provide mechanistic information on endocrine system function. Older subchronic rodents studies may or may not have weighed or examined all accessory sex tissues separately
Developmental neurotoxicity (DNT), rat	870.6300	426	Following perinatal exposure (GD6-PND21), measurement of: • Sexual maturity (VO and PPS) • Day of eye opening • Behavioural ontogeny, neurobehavioral assessment, motor activity and coordination, acoustic startle, learning and memory • Brain wt, histopath and morphometry • Serum thyroid hormones (optional)	The DNT exposure examines effects resultant to perinatal exposure only, as dosing does not continue through sexual maturation. Acoustic startle is a relatively insensitive technique to assess hearing changes in rats due to moderate perturbations in thyroid homeostasis. Neurobehavioral endpoints and learning and memory are more sensitive, but may miss minor changes in thyroid function. Similarly, brain morphometry changes may only be sensitive to severe thyroid hormone deprivation; myelin staining, which may aid in detection of thyroid-related neurological changes, is optional. Serum thyroid hormone measurement is not required and not always available
Combined chronic and oncogenicity study, rat Oncogenicity study, mouse Chronic dog (1 or 2 year exposure)	870.4100 870.4200 870.4300 870.4200 870.4100	452 451 453 451 452	Following prolonged exposure (1–2 years), measurement of: • Weights (liver, kidneys, adrenals, gonads, brain) • Gross pathology and histopathology of numerous tissues, including endocrine-relevant tissues (pituitary, brain, thyroid/parathyroid, adrenals, testis, ovary, uterus, epididymis, prostate, seminal vesicles, and female mammary gland) in male and female animals	Weights, gross pathology, and histopathology, while providing valuable information, do not provide mechanistic information on endocrine system function. Older studies may or may not have weighed or examined all accessory sex tissues separately
Developmental toxicity, rodent/ rabbit	870.3700 (83–3 before 1998) 870.3550 870.3650	414 421 422	Following exposure of dams from implantation to the day prior to expected parturition, measurement of: • Maintenance of pregnancy • Uterus wt • # of implants and corpora lutea • Viability, litter size, sex ratio, fetal BW • Fetal examinations (external, visceral, and skeletal)	Though some aspects of endocrine and reproductive function are assessed by this study design, endocrine assessment of the offspring (F1 generation) is limited
Multigenerational study, rodent	870.3800 (83–4 before 1998)	416	Exposure of parental (P) generation before and during mating, during pregnancy, and through the weaning of the F1 generation. F1 generation is then exposed during growth, mating, pregnancy, generation of the F2 generation, through F2 weaning • Evaluation of reproductive performance (mating, fertility, time to mating, gestation length, difficulty of parturition) • Litter parameters (offspring viability, litter size, pup body weights and sex ratio) • Histopathology of endocrine-relevant tissues (vagina, uterus, ovaries, testes, epididymides, seminal vesicles, prostate, pituitary and target organs)	Studies run prior to 1998 are unlikely to report all reproductive and accessory sex tissue organ wts, estrous cyclicity, ovarian follicle counts, sperm parameters, puberty onset, thyroid wt/histopath, adrenal wt/histopathology and/or AGD (optionally triggered in F2 offspring)

Table 6. Triadimefon: Endocrine-related HTS bioactivity data.

Triadimefon Screening Information *Estimated cytotoxicity limit = 1.68 μM*			
Endocrine activity hypothesis (positives/total # assays)	Assay Endpoint ID	AC50 (μM)	AUC model score
Steroidogenesis (2/2)	NVS_ADME_hCYP19A1	2.07	NA‡
	Tox21_Aromatase_Inhibition	36.84	
Estrogenic (4/15)	ACEA_T47D_80hr_Positive	7.94	0.0095 (negative)
	ATG_ERa_TRANS_up	16.46	
	ATG_ERE_CIS_up	18.08	
	OT_ER_ERaERb_0480*	24.91	
Anti-estrogenic (1/13)	OT_ER_ERaERb_0480*	24.91	0
Androgenic (2/10)	NVS_NR_hAR†	11.05	0
	NVS_NR_cAR	19.63	
Anti-androgenic (2/8)	NVS_NR_hAR	11.05	0
	NVS_NR_cAR	19.63	
Thyroid receptor agonist activity (0/3)	Negative	NA	NA
Thyroid receptor antagonist activity (0/2)	Negative	NA	NA
Hepatic catabolism and thyroid hormone clearance (27/77)	ATG_Ahr_CIS_up	33.88	NA
	ATG_PBREM_CIS_up	16.00	
	ATG_PPARg_TRANS_up	31.52	
	ATG_PPRE_CIS_up	55.54	
	ATG_PXR_TRANS_up	17.79	
	ATG_PXRE_CIS_up	7.10	
	CLZD_CYP1A1_24	12.03	
	CLZD_CYP1A1_48	11.32	
	CLZD_CYP1A1_6	8.89	
	CLZD_CYP1A2_24	11.94	
	CLZD_CYP1A2_48	11.90	
	CLZD_CYP2B6_24	8.89	
	CLZD_CYP2B6_48	7.73	
	CLZD_CYP2B6_6	7.04	
	CLZD_CYP3A4_24	5.82	
	CLZD_CYP3A4_48	5.67	
	CLZD_SULT2A1_48	8.29	
	CLZD_UGT1A1_24	9.66	
	NVS_ADME_hCYP1A1	2.09	
	NVS_ADME_hCYP2C19	1.18	
	NVS_ADME_hCYP2C9	7.63	
	NVS_ADME_hCYP3A4	4.89	
	NVS_ADME_rCYP1A1	16.37	
	NVS_ADME_rCYP2B1	0.51	
	NVS_ADME_rCYP3A1	8.94	
	NVS_NR_hPXR	3.68	
	Tox21_AhR	88.51	

*This assay may inform E and anti-E models. This assay dose-response is also annotated with caution flag 11: "Borderline active," as the highest concentration tested which may decrease the reliability of this AC50 value.
†These assays may inform A and anti-A models.
‡NA indicates there was no AUC model score available for the endocrine hypothesis.

manuscript focuses on mammalian models, but further interpretation of the results of all three triazoles in the FSTRA and AMA is publicly available (USEPA 2015a), and a brief overview is provided in Supplemental File 4.

Triadimefon

Triadimefon does not appear to interact directly with the ER, AR, or TR pathways (Table 9) (Willoughby 2012a–c). The EDSP Tier 1 assays for triadimefon suggest high-dose effects on steroidogenesis that include inhibition of aromatase (50% inhibitory concentration [IC_{50}] range of 1.7–32 μM) and decreased testosterone levels (at 10–100 μM) in the H295R cell steroidogenesis assays (Table 9) (Goetz et al. 2009). Although this direction of change is inconsistent with increased serum testosterone observed in rats *in vivo* in a male pubertal study design (Goetz et al. 2007), the combined results for triadimefon suggest some potential for disruption of steroidogenesis rather than a direct impact on AR signaling, as the Hershberger assay was negative (Davis 2011). Changes in estradiol production in the H295R assay were not dose-responsive (Goetz et al. 2009), and effects on relative ovary weight in a pubertal study design failed to correspond to changes in histopathology or serum estradiol, although vaginal opening (VO) was delayed and estrous cyclicity was transiently disrupted (Rockett et al. 2006). Neurotoxicity, liver toxicity, BW decreases and other signs of systemic toxicity likely represent the primary toxicities of triadimefon, and the inconsistent direction of change in testosterone between *in vitro* and *in vivo* models used to satisfy the data requirements of EDSP Tier 1 screening, along with any minor effects on estrogen-sensitive markers, may be a result of the interaction of these toxicities at high doses *in vivo*.

The AMA (Table 9b in Supplemental File 4), used to determine potential thyroid effects, was waived based on both OSRI and guideline toxicology studies that already demonstrated high-dose effects on thyroid histopathology and serum thyroid hormone concentrations in rodents. These high-dose effects are considered sequelae to primary effects on the liver (USEPA 2015a), as rat thyroid perturbation occurred at doses associated with upregulated hepatic catabolism and thyroid hormone clearance based on co-

Table 7. Propiconazole: Endocrine-related HTS bioactivity data.

Propiconazole Screening Information *Estimated cytotoxicity limit = 4.6 μM*			
Endocrine activity (positives/total # assays)	Assay	Propiconazole (AC50, uM)	AUC model score
Steroidogenesis (2/2)	NVS_ADME_hCYP19A1	2.24	NA
	Tox21_Aromatase_Inhibition	23.76	
Estrogenic (3/16)	ACEA_T47D_80hr_Negative	82.41	0
	ATG_ERa_TRANS_up	26.14	
	ATG_ERE_CIS_up	1.65	
Anti-estrogenic (2/14)	Tox21_ERa_BLA_Antagonist_ratio	71.27‡	0
	Tox21_ERa_LUC_BG1_Antagonist	56.36	
Androgenic (3/9)	NVS_NR_cAR†	12.89	0
	NVS_NR_hAR†	19.88	
	OT_AR_ARSRC1_0960†	35.39	
Anti-androgenic (5/8)	NVS_NR_cAR†	12.89	0.11
	NVS_NR_hAR†	19.88	
	OT_AR_ARSRC1_0960†	35.39	
	Tox21_AR_BLA_Antagonist_ratio	33.48	
Parallel viability assays	Tox21_AR_BLA_Antagonist_viability	51.75	
	Tox21_AR_LUC_MDAKB2_Antagonist	69.99¶	
Thyroid Receptor Agonist Activity (0/3)	Negative	NA	NA
Thyroid Receptor Antagonist Activity (1/2)	Tox21_TR_LUC_GH3_Antagonist	54.73	NA
Hepatic catabolism (27/76)	ATG_Ahr_CIS_up	23.04	NA
	ATG_PBREM_CIS_up	48.23	
	ATG_PXRE_CIS_up	5.54	
	CLZD_ABCB1_24	7.96	
	CLZD_ABCB1_48	6.23	
	CLZD_ABCG2_48	8.32	
	CLZD_CYP1A1_24	11.18	
	CLZD_CYP1A1_48	7.36	
	CLZD_CYP1A1_6	8.70	
	CLZD_CYP1A2_24	5.91	
	CLZD_CYP1A2_48	9.18	
	CLZD_CYP1A2_6	5.94	NA
	CLZD_CYP2B6_24	0.49	
	CLZD_CYP2B6_48	0.46	
	CLZD_CYP2B6_6	0.47	
	CLZD_CYP3A4_24	0.53	
	CLZD_CYP3A4_48	2.96	
	CLZD_SLCO1B1_48	0.034	
	CLZD_SULT2A1_48	7.44	
	NVS_ADME_hCYP1A1	9.01	
	NVS_ADME_hCYP2B6	2.63	
	NVS_ADME_hCYP2C19	0.57	
	NVS_ADME_hCYP2C9	4.41	
	NVS_ADME_rCYP2B1	0.38	
	NVS_ADME_rCYP3A1	1.95	
	NVS_NR_hCAR_Antagonist	32.03	
	Tox21_AhR	23.27	

†These assays may inform androgenic and anti-androgenic models

‡The Tox21_ERa_BLA_Antagonist_ratio assay dose-response is annotated with caution flag 12: "Borderline inactive," decreasing the reliability of this AC50 value.

¶The Tox21_AR_LUC_MDAKB2_Antagonist assay dose-response curve is annotated with caution flag 6: "Only highest conc above baseline, active," indicating that only the maximum concentration tested exceeded the range of baseline values.

incidence of increased liver weight and hepatic centrilobular hypertrophy. Taken together, these potentially endocrine-related effects on the thyroid appear to require a dose that exceeds the threshold for induction of effects in the liver and other markers of systemic toxicity.

Propiconazole

The EDSP Tier 1 assays and published OSRI used to evaluate propiconazole identified effects at high *in vitro* concentrations on steroidogenesis that did not manifest consistent effects in *in vivo* studies in mammalian species in which the full steroidogenesis pathways and control mechanisms were present (Table 10). For example, a series of *in vitro* studies showed inhibition of aromatase by propiconazole with IC_{50} values of 0.968–8.25 μM, and steroidogenesis assays in H295R cells showed decreased testosterone and estradiol concentrations at ≥1 μM, but there were no consistent patterns of change that would reflect altered steroidogenesis in the variety of *in vivo* EDSP Tier 1 and OSRI studies (Table 10). Similarly, a series of *in vitro* assays suggests anti-androgenic potential of propiconazole at high concentrations, likely confounded by cytotoxicity, but corroborative anti-androgenic effects in the *in vivo* assays were not observed. Overall, liver and systemic toxicity appear to be the primary toxicities for propiconazole that are evident from mammalian *in vitro* and *in vivo* EDSP Tier 1 screening assays and OSRI.

Table 8. Myclobutanil: endocrine-related HTS bioactivity data.

Myclobutanil Screening Information *Estimated cytotoxicity limit = 4.69 μM*			
Endocrine activity (positives/total # assays)	Assay ID	AC50 (μM)	AUC model score
Steroidogenesis (2/2)	NVS_ADME_hCYP19A1	0.672	NA
	Tox21_Aromatase_Inhibition	5.16	
Estrogenic (2/15)	ATG_ERa_TRANS_up	34.67*	0
	ATG_ERE_CIS_up	5.11†	
Anti-estrogenic (0/13)	Negative	NA	0
Androgenic (0/8)	Negative	NA	0
Anti-androgenic (0/6)	Negative	NA	0
Thyroid receptor activity (0/4)	Negative	NA	NA
Hepatic catabolism (19/79)	ATG_PXRE_CIS	13.86	NA
	CLZD_CYP1A1_48	6.73	
	CLZD_CYP1A2_24	5.99	
	CLZD_CYP1A2_48	11.84	
	CLZD_CYP2B6_24	11.28	
	CLZD_CYP2B6_48	7.26	
	CLZD_CYP2B6_6	4.15	
	CLZD_CYP2C9_48	3.91	
	CLZD_CYP3A4_24	6.07	
	CLZD_CYP3A4_48	5.93	
	CLZD_CYP3A4_6	5.24	
	CLZD_UGT1A1_24	14.60	
	NVS_ADME_rCYP3A1	3.69	
	NVS_ADME_rCYP2B1	1.52	
	NVS_ADME_hCYP2B6	5.66	
	NVS_ADME_hCYP1A1	10.66	
	NVS_ADME_rCYP3A2	2.77	
	NVS_ADME_hCYP2C19	0.58	

*The dose–response curve information for ATG_ERE_CIS_up is accompanied by 1 caution flag: 16: "Hit-call potentially confounded by overfitting."

†The dose–response curve information for ATG_ERa_TRANS_up is accompanied by 3 caution flags that suggest decreased relevance (6: "Only highest conc above baseline, active"; 11: "borderline active; and, 16: "Hit-call potentially confounded by overfitting").

Myclobutanil

The studies that satisfied the EDSP Tier 1 data requirements for myclobutanil suggest *in vitro* aromatase inhibition (an IC_{50} range of 0.1–47 μM) and decreased estradiol (≥1 μM) and testosterone production (1—100 μM) in *in vitro* steroidogenesis assays, with no evidence of any direct interaction with ER, AR or TR pathways (Table 11). Given the lack of evidence of anti-estrogenicity in EDSP Tier 1 *in vivo* study data, the relevance of decreased estradiol production *in vitro* is not supported by higher tier data. More specifically, the uterotrophic assay (Marty & Brooks, 2011) and the female pubertal assay (Marty et al. 2011) were negative. Myclobutanil was negative in the Hershberger assay (Marty et al. 2011), suggesting a lack of direct effect on the AR pathway. In the male pubertal assay, a significant decrease in testosterone levels correlated with a decrease in multiple androgen-dependent tissue weights and a slight delay in preputial separation (PPS) (Marty et al. 2011), but at doses that increased liver weights and hypertrophy. Alteration of testosterone homeostasis in mammals may also be influenced via activation of CAR, PXR and subsequent enzyme induction in the liver, as demonstrated by all three of these triazole fungicides (Goetz & Dix, 2009; Goetz et al. 2009). Therefore, although myclobutanil altered steroidogenesis *in vitro* (likely via aromatase inhibition), the effects on androgen-sensitive endpoints *in vivo* may result from more than one MOA, possibly including altered liver-mediated steroid hormone metabolism.

Thyroid-related changes *in vivo* were observed at high-dose levels in the male pubertal assay (Marty et al. 2011) and a published study (Wolf et al. 2006) (Table 11 and Supplemental File 5) in the presence of liver toxicity. These effects on serum thyroid hormones and thyroid histology likely occurred via a well-recognized indirect mechanism involving liver enzyme induction and subsequently increased clearance of thyroid hormones (Barter & Klaassen 1992; Hood et al. 1999; Vansell & Klaassen 2002).

Overall, myclobutanil altered *in vitro* steroidogenesis via aromatase inhibition, but *in vivo* changes in androgen-sensitive endpoints occurred in the presence of systemic toxicity, including increased liver weight and hepatocellular hypertrophy suggestive of enhanced enzyme induction in the liver and altered steroid hormone homeostasis.

Guideline toxicology studies: line of evidence 3

Guideline toxicology study results for triadimefon, propiconazole, and myclobutanil are presented in Tables 12–14. Detailed narrative reviews of this information are provided for each chemical in Supplemental File 5.

Triadimefon

Available 40 CFR Part 158 guideline toxicology study information suggests high-dose effects of triadimefon on male mating behavior in rats in multi-generation studies, possibly resultant

Table 9. Triadimefon: mammalian EDSP Tier 1 assay results plus published OSRI.

Study	Methods	Concentrations tested	E	Anti-E	A	Anti-A	S	Anti-T	Additional findings
ER binding assay OCSPP 890.1250 (Willoughby 2012b)	ER binding *in vitro*	10^{-10} to 10^{-4} M	Non-interacting	Non-interacting					
AR binding assay (OCSPP 890.1150) (Willoughby 2012a)	AR binding *in vitro* using rat prostate	10^{-11} to 10^{-3} M, precipitation at 10^{-3}			Non-interacting	Non-interacting			
ER-TA OCSPP 890.1300 (Willoughby 2012c)	ER transactivation *in vitro* in HeLa cells	$10^{-10.3}$ to 10^{-4} M	Activates transcription at 30 – 100 μM by 18–30%						
ER-TA and AR-TA. OSRI cited: (Kojima et al. 2004)	CHO cells with hERα or hERβ or hAR and luciferase reporter (± 17β-estradiol or DHT)	10 nM to 10 μM	No Activation	No Inhib	No Activation	No Inhib			
Aromatase inhibition *Waived*; USEPA (2007a) validation study (OCSPP 890.1200)	Aromatase inhib *in vitro*	10^{-4} to 10^{-8} M		May have an anti-E effect	May have an A effect		Inhib aromatase with IC_{50} = 1.71 μM		
Aromatase inhibition OSRI cited: (Vingaard et al. 2000)	Aromatase inhib with human placental microsomes	1 – 100 μM		May have an anti-E effect.	May have an A effect.		Inhib aromatase with IC50 = 32 μM		
Aromatase inhibition OSRI cited (Ohno et al. 2004)	KGN cells incubated with androstenedione; measured levels of estrone	500 pM to 50 μM		May have an anti-E effect	May have an A effect		Inhibits aromatase with IC_{50} = 3.59 μM		
Aromatase inhibition OSRI cited (Trosken et al. 2004)	Aromatase inhib with human recombinant CYP19	A range covering three orders of magnitude		May have an anti-E effect.	May have an A effect		Inhibits aromatase with IC_{50} = 17.5 μM		
Steroidogenesis *Waived* (OCSPP 890.1550)	H295R cell line assay	NA							
Steroidogenesis, OSRI cited: (Goetz et al. 2009)	H295R cell line	1, 3, 10, 30 and 100 μM	↑ estradiol at 3 μM; no estradiol effect at >3 μM (not dose-responsive)			↓ testosterone by 20–80% at 10 – 100 μM	Data suggest effects on *in vitro* S		
	Testis organ culture	1, 10 and 100 μM				Negative	↓ testosterone and androstenedione production and ↑ 17α-hydroxyprogesterone and progesterone at 10–100 μM (consistent with CYP17A1 inhib)		
	In vivo 30 d dietary exposure in ♂ Wistar rats	126 mkd	No change in serum estradiol		↑ serum and intratesticular testosterone	Minor ↓ in epididymis wt	Dysregulation of A and Anti-A endpoints interpreted to be related to S or systemic toxicity		↓ BW and ↑ liver wt; small ↓ in pituitary wt

(*continued*)

Table 9. Continued

Study	Methods	Concentrations tested	E	Anti-E	A	Anti-A	S	Anti-T	Additional findings
Uterotrophic Assay *Waived* (OCSPP 890.1600)	*In vivo* uterine wt response to a minimum of 3 d of exposure	NA (OSRI; see Rockett et al. 2006 and multi-generation studies)							
Female Pubertal Assay (OCSPP 890.1450) *Waived*	*In vivo* pubertal landmarks	NA (OSRI; see Rockett et al. 2006 and multi-generation studies)							
Female reproductive development study OSRI cited: (Rockett et al. 2006)	*In vivo* pubertal landmarks following perinatal and PND (PND22-PND99) exposure	100, 500 and 1800 ppm Depending on maternal age, doses were 7.9–17.8, 35.9–94.1 and 94.3–300.1 mkd Depending on F1 age, doses were 7.3–14.9, 39.1–76.8 and 139–267 mkd	↑ rel ovary wt with no effects on histopath at 1800 ppm in F1; no effects on serum estradiol	Delayed VO at 1800 ppm in F1; disrupted estrous cyclicity at weeks 5 and 6 only at 1800 ppm in F1; no effects on serum estradiol			Effects on E status suggested (see anti-E column) No effect on: serum estradiol levels	Mild follicular cell hypertrophy in T at (1800 ppm) 136 mkd in 2/4 rats	↓ maternal (gestation and week 1 and −2 lactation) and F1 (weaning to necropsy) feed intake at 1800 ppm; ↑ rel liver wt and ↑ incidence of panlobular hypertrophy at 1800 ppm; ↓ BW for PND99 animals at 1800 ppm
Hershberger Assay (OCSPP 890.1400) (Davis 2011)	Short-term effects on wts of 5 androgen-sensitive organs in rats (± testosterone admin.)	25, 50 and 100 mkd; 10 d post-exposure; agonist and antagonist (with admin of testosterone proprionate, TP)			Agonist mode: *Negative. Findings:* ↑ ventral prostate wt by 29% at 100 mkd; no effect on glans penis, Cowper's gland, levator ani plus bulbocavernous muscle complex (LABC) or seminal vesicles.	Antagonist mode: *Negative. Findings:* co-admin with TP at 100 mkd ↓ BWs; ↓ LABC wt 14%; no effect on glans penis, Cowper's gland, ventral prostate, seminal vesicle, or liver wt			↓ BWG at 50 and 100 mkd; ↑ abs (20%) and rel liver wt at 100 mkd
Male Pubertal Assay *Waived* (OCSPP 890.1500)	*In vivo* pubertal landmarks	NA (OSRI; see Goetz et al. 2007 and multi-generation studies)							
OSRI cited: Goetz et al. (2007)*	♂ repro dev landmarks following perinatal and PND 22-92 exposure	100, 500 and 1800 ppm mkd not reported; likely top dose (1800 ppm) ranged 94.3–300.1 mkd (see Rockett et al. 2006)			• ↑ testes abs/rel wt at 500 ppm on PND22, and at 100 and 500 ppm on PND50 (no high dose ♂ available), and ↑ rel testis wt only for 1800 ppm on PND92 in F1 • ↑ AGD at 1800 ppm in F1 ♂; • ↑ serum testosterone at 500 and 1800 ppm in F1 ♂; no effect on testes histopath	• Delayed PPS at 1800 ppm in F1 ♂ • ↓ mating by F1 ♂ at 1800 ppm with no sperm effects • Failure to inseminate resulted in inability to measure fertility		No effects on T wt or histopath. T4 ↓ at 1,800 ppm on PND 92	• Decreased pup survival (↓47.37%) at 1800 ppm 20–30% ↓ BW (PND1-92) at 1800 ppm. ↓ rel brain wt (PND92) at 1800 ppm • ↓ feed consumption in F1 ♂for much of the study • ↑ 20% rel liver wt at PND92 with 1800 ppm • No copulatory plugs for any of the F1 1800 ppm matings.

(*continued*)

Table 9. Continued

Study	Methods	Concentrations tested	E	Anti-E	A	Anti-A	S	Anti-T	Additional findings
OSRI cited: Tully et al. (2006)	Testis and hepatic biomarkers following a 14-d exposure in ♂rats	0, 5, 50 and 115 mkd			No effect on serum testosterone; no effects on testis wt or histopath. No effect on sperm morphology or motility		No effects on T histopath	Centrilobular hypertrophy and ↑rel liver wt (9–20%) at 50 and 115 mkd	
OSRI cited: Wolf et al. (2006)	Markers of T disruption; dietary exposure in ♂Wistar rats for 4, 30, or 90 d	100, 500 and 1800 ppm						• ↓ T4 (4, 30 d exposure), ↓ T3 (4, 30 d exposure,) and ↓ TSH (4 d exposure) with 1800 ppm; • T follicular cell hypertrophy, colloid depletion, and cell prolif. with at 30 d but not 90 d at 1800 ppm	
OSRI cited: Hester and Nesnow (2008)	Markers of T disruption; dietary exposure to ♂Wistar rats for 30 or 90 d	1800 ppm in feed (106.7 mkd)						↑ PPARγ, markers of oxidative stress (CYP activation), cell proliferation and migration, and fatty acid biosynthesis in T tissue	

*Goetz et al. (2007): rel ventral prostate wt was ↑ by 2–3% at 100 and 500 ppm, but not at 1800 ppm in F1 ♂ at PND92. Rel seminal vesicle wt was ↑ at PND92 at 500 ppm, but not at 1800 ppm. With 1800 ppm exposure, the abs seminal vesicle wt was ↓, but with no effects on rel seminal vesicle wt. Considering the minor level of changes, inconsistent dose-response relationships, and lack of micropathology, we did not consider these findings relevant.

Table 10. Propiconazole: mammalian EDSP Tier 1 assay results plus published OSRI.

Study	Methods	Concentrations tested	E	Anti-E	A	Anti-A	S	Anti-T	Other info., additional findings
ER binding assay OCSPP 890.1250 (Willoughby 2012d, e)	ER binding *in vitro* using rat uterine cytosol	10^{-10} to 10^{-3} M	Non-interacting	Non-interacting					
AR binding assay (OCSPP 890.1150) *Waived*	AR binding *in vitro* using rat prostate	NA							
AR binding assay OSRI cited: Bauer et al. (2002)	Recombinant human AR on microtiter plates	10–200 μM			Ki = 95.9 μM	Ki = 95.9 μM			
AR binding assay OSRI cited: Kojima et al. (2001)	AR reporter assay in CHO cells (agonist and antagonist assays)	0.01–10 μM.			Non-interacting	6.2 μM = 20% inhib			
AR Binding Assay OSRI cited: Vinggaard et al. (2008).	Human AR reporter assay in CHO cells (antagonist assay)	1, 3, 10 and 30 μM				3 μM < IC25 < 10 μM			
AR Binding Assay OSRI cited: Kjaerstad et al. (2007)	Human AR reporter assay in CHO cells (antagonist assay)	0.025–50 μM				LOEC = 25 μM			
ER-TA OCSPP 890.1300 *Waived*	ER transactivation *in vitro* in HeLa cells	NA							
ER-TA. OSRI cited: (Kjaerstad et al. 2007)	Cell proliferation in MCF-7 cells by ELISA	1 nM to 150 μM	AC50 > 25 μM	IC50 = 52 uM					
ER-TA. OSRI cited: (Kojima et al. 2004)	CHO cells with ERα or ERβ and luciferase reporter (± estradiol)	10 nM to 10 μM	No activation	No inhibition					
ER-TA. OSRI cited: (Hurst and Sheehan, 2003)	yeast cells with hERα and Lac-Z reporter	Inappropriate test system (yeast) for a fungicide	Not applicable	Not applicable					
Aromatase inhibition *Waived*; (OCSPP 890.1200)	human recombinant CYP19 incubated with ^{3}H-androstendione	NA							
Aromatase inhibition OSRI cited: (Ohno et al. 2004)	KGN cells incubated with androstenedione; measured levels of estrone	100 pM–10 μM		May have an anti-E effect	May have an A effect		Inhibits aromatase with IC_{50} = 0.968 μM		
Aromatase inhibition OSRI cited: (five other studies)	Aromatase inhib assay; Various methods	Multiple; up to 100 μM		May have an anti-E effect	May have an A effect		IC50 values = 3.2–8.25 μM		Laville et al. (2006), Vingaard et al. (2000), Trosken et al. (2004, 2006b), Sanderson et al. (2002)
Steroidogenesis *Waived* (OCSPP 890.1550)	H295R cell line assay, measure 17β-estradiol and testosterone content	NA							
Steroidogenesis OSRI cited: Goetz et al. (2009)	H295R cell line assay, measure 17β-estradiol and testosterone	1–100 μM		Possible anti-E effect at high concentrations		Possible anti-A effect at high concentrations	↑ Estradiol at 1–3 μM ↓ Estradiol, testosterone and progesterone at ≥10 μM		
Steroidogenesis OSRI cited: Kjaerstad et al. (2007)	H295R cell line assay, measure 17β-estradiol and testosterone	0.1–30 μM		Possible anti-E effect at high concentrations		Possible anti-A effect at high concentrations	↓ Estradiol, testosterone at ≥1 μM ↑ Progesterone at ≥1 μM		
Uterotrophic assay (OCSPP 890.1600) Coder (2012)	*In vivo* uterine wt response to a minimum of 3 d of exposure	0, 175, 400 and 500 mkd in OVX rats	Negative						

(*continued*)

Table 10. Continued

Study	Methods	Concentrations tested	E	Anti-E	A	Anti-A	S	Anti-T	Other info., additional findings
Female pubertal assay (OCSPP 890.1450) *Waived*	*In vivo* pubertal landmarks	NA							
Female reproductive development study; female pubertal assay OSRI cited: (Rockett et al. 2006)	*In vivo* pubertal landmarks following perinatal and PND (GD6-PND99) exposure	0, 100, 500 and 2500 ppm in diet	No effects that would indicate an E effect, including • AGD in ♀ • Litter size • Gender ratio • Age at VO • Estrous cycling • Endo-crine sensitive organs wts • Histology • Serum estradiol	No effects that would indicated an anti-E effect (see prior column)			No effects on serum estradiol levels	No effects that would indicate an anti-T effect: • T wt • T micropath	
Hershberger Assay (OCSPP 890.1400) *Waived*	Short-term effects on wts of 5 androgen-sensitive organs in rats (± testosterone admin)	NA							
Hershberger Assay OSRI cited: Kjaerstad et al. (2007) and Taxvig et al. (2008)	Anti-A Hershberger assay; castrated ♂ rats treated with testostero-ne + increasing doses of propiconazole	50, 100 and 150 mkd, orally for 7 d			Agonist mode: *Negative* (see Goetz et al. 2007 below, under male pubertal assay)	Antagonist mode: *Negative. Findings:* No effect on wt of prostate, seminal vesicles, LABC, bulbourethral gland, pituitary		No effect on T4 levels or T wt	No effect on LH levels; ↑ FSH at 150 mkd
Hershberger Assay OSRI cited: Taxvig et al. (2008)	2nd study: pregnant rats treated GD7–GD21	50 mkd	No effect on implantations or AGD in fetuses	No effect on implantations or AGD in fetuses	No effect on implantations or AGD in fetuses	No effect on implantations or AGD in fetuses	*Negative*. No effect on testosterone, progesterone or estradiol in dams; No effect on hormones in tissues of fetuses (estradiol, testosterone, progesterone)		↑ 17α-hydroxy-progesterone in serum of dams
Male pubertal assay *Waived* (OCSPP 890.1500)	*In vivo* pubertal landmarks	NA							
Male Pubertal Assay *OSRI cited:* Goetz et al. (2007)*[b]	♂ repro dev landmarks following perinatal and PND (GD6 – PND92) exposure to Wistar rats	0, 100, 500 and 2500 ppm in diet			↑ AGD at 2500 ppm in F1 ♂;. no effect in ♀ or in several other similar studies (Taxvig et al. 2008)	*Negative*. No effects on: age at PPS, wt of prostate, seminal vesicles, epididymis, testis wt*; sperm parameters; micropathology of androgen-sensitive organs	*Negative*. No consistent effects on serum E levels or testosterone† in F1 ♂	No effects on T wt, histopath or T3, T4 and TSH levels in F1 ♂, PND 92	No effects on repro success of treated F1 ♂ paired with untreated ♀ (any dose level)
Male Pubertal Assay *OSRI cited:* Tully et al. (2006)	Testis and hepatic biomarkers following a 14-d exposure in ♂ SD rats	0, 10, 75 and 150 mkd			No effects on prostate, seminal vesicle, or testis wts. No effects on histopath, sperm morphology or sperm motility.	No effects on prostate, seminal vesicle, or testis wts. No effects on histopath, sperm morphology or sperm motility.	*Negative*. No effects on serum estradiol or testosterone, or on LH or FSH levels.	No effects on T histopath	↑ liver wts (13–19%), 75and 150 mkd; hepatocyte hypertrophy
Thyroid-Related Tests in Mammals *OSRI cited, supplemental to OCSPP 890.1100:* Wolf et al. 2006	Markers of T disruption; dietary exposure in ♂ Wistar rats for 4, 30, or 90d	0, 100, 500 and 2500 ppm						↓ serum T4 on ds 4 and 30 (2500 ppm), but T4, T3 and TSH normal by d 90. No effect on T histology	↑ UDPGT activity in liver (2500 ppm)

*Goetz et al. (2007): Testis wt was determined at PND1, 22, 50 and 92, and was ↑ only on PND22 at 2500 ppm (rel wt alone) and on PND50 at 500 ppm (abs, rel wt) and 2500 ppm (rel wt alone), but not at any other intervals. Considering the lack of consistency in this study and across other studies, plus the changes in relative wt secondary to lower BW, this was not considered evidence of a biologically significant change.

†Goetz et al. (2007): Testosterone levels in F1 ♂ were ↑ on PND92 at 500 and 2500 ppm, but not on PND50 or in other studies. Considering the lack of consistency, this was not considered evidence of a biologically significant change.

Table 11. Myclobutanil: mammalian EDSP Tier 1 assay results plus published OSRI.

Study	Methods	Concentrations tested	E	Anti-E	A	Anti-A	S	Anti-T	Additional findings
ER Binding Assay OCSPP 890.1250 (LeBaron et al. 2011a)	ER binding *in vitro*	10^{-10} to 10^{-3} M	Non-interacting	Non-interacting					
AR Binding Assay (OCSPP 890.1150) (LeBaron et al. 2011b),	AR binding *in vitro* using rat prostate	10^{-10} to 10^{-3} M			Equivocal at 1mM	Equivocal at 1mM			
ER-TA OCSPP 890.1300 (LeBaron et al. 2011c)	ER transactivation *in vitro* in HeLa cells	10^{-10} to 10^{-4} M	*No activation*						
ER-TA. OSRI cited: (Hurst & Sheehan 2003)	Yeast cells with hERα and Lac-Z reporter	Inappropriate test system (yeast) for a fungicide.	Not applicable	Not applicable					
Aromatase inhibition (OCSPP 890.1200) (Coady & Sosinski 2011)	Aromatase inhib *in vitro*	10^{-10} to 10^{-8} M		May have an anti-E effect	May have an A effect		Inhib aromatase with IC_{50}=0.1 μM		
Aromatase inhibition OSRI cited: (Trosken et al. 2004)	A fluorimetric assay based on human recombinant CYP19 enzyme with dibenzyl-fluorescein (DBF) as a substrate was used to compare the inhibitory potency	A range covering 3 orders of magnitude		May have an anti-E effect	May have an A effect		Inhib aromatase with IC_{50}=0.47 μM		
Aromatase inhibition OSRI cited: (Trosken et al. 2006b)	Microsomes (''Supersomes'') containing human aromatase (CYP19) coex-pressed with human cyto-chrome P450 reductase (baculo-virus/insect cell expressed) were from BD Gentest. Testosterone was utilized as the substrate	Not provided		May have an anti-E effect	May have an A effect		Inhib aromatase with IC_{50} = 47μM		
Steroidogenesis (OCSPP 890.1550) (LeBaron et al. 2011d)	H295R cell line assay	10^{-10} to 10^{-4} M		↓estradiol at the three highest con-centrations tested (1, 10 and 100 μM).		↓testosterone production only at the assay limit concentration (100μM).	Data suggest effects on *in vitro* S (≥1 μM)		
Steroidogenesis, OSRI cited: (Goetz et al. 2009)	H295R cell line	1, 3, 10, 30 and 100 μM		↓ estradiol ≥3 μM		↓ testosterone ≥ 1μM	Data suggest effects on *in vitro* S (≥1 μM)		
Uterotrophic Assay (OCSPP 890.1600) (Marty & Brooks 2011)	*In vivo* uterine wt response to a minimum of 3 d of exposure	0, 50, 150 and 475 mkd	**Negative**						
Female Pubertal Assay (OCSPP 890.1450) (Marty et al. 2011a)	*In vivo* pubertal landmarks	0, 50, 200 and 400 mkd	• *Negative* • No effects that would indicate an E effect, including • Age/BW at VO • Estrous cycling, Pituitary, ovarian or uterine wts • Histology • Serum estradiol	*Negative*. No effect that would indi-cate an anti-E effect (see prior column)			*Negative*. No effect that would indicate altered estrogen synthesis (see ''estrogenic'' end-points); also no effect on: adrenal wts	*Negative*. No effects that would indicate an anti-T effect: • T wt • T micropath • T4 or TSH levels	Liver: • ↑ Abs/rel wts at 200 and 400 mkd (18–41%) • Increased alanine amino-transferase and gamma glutamyl *trans*-peptidase at 400 mkd • Slight hepatocellular hypertrophy and ↑ mitotic figures at 200 and 400 mkd

(*continued*)

Table 11. Continued

Study	Methods	Concentrations tested	E	Anti-E	A	Anti-A	S	Anti-T	Additional findings
Female reproductive development study OSRI cited: (Rockett et al. 2006)	*In vivo* pubertal landmarks following perinatal and PND (GD6-PND99) exposure	0, 100, 500 and 2000 ppm in diet (mkd ranges: 0, 8–19.1, 38.7—93.8 and 141.3–347.3)	Negative. No effects on • Serum E levels • Estrous cycling • Uterine wt • Ovarian histopath	No effects on: • Serum estradiol levels • Estrous cycling • Uterine wt • Ovarian histopath Effects: • ↑AGD and delayed VO (+2.2 ds)in ♀ pups and ↑ovarian wt (45%) at 2000 ppm			Effects on E status suggested (see anti-Ecolumn) No effect on: serum estradiol levels	No effects that would indicate an anti-T effect: • T wt • T histopath	• ↓ feed intake (wk 2 lactation) at 2000 ppm • At PND 0 and VO, there were no significant differences in BW • No effects on rel brain hypo-thalamus and liver wts
Hershberger Assay (OCSPP 890.1400) (Marty & Marshall 2011)	Short-term effects on wts of 5 androgen-sensitive organs in rats (± testosterone admin.)	0,25,125 and 500 mkd; 10 d post-exposure; agonist and antagonist (with admin of testosterone proprionate)			Agonist mode: *Negative.* No effects on LABC, glans penis, seminal vesicle, ventral prostate or Cowper's gland	Antagonist mode: *Negative.* No effects on glans penis, Cowper's gland, ventral prostate, or seminal vesicle wts. ↓ only in LABC wt (20%) at 500 mkd			• ↓ BWG (62–81%) (TD1-4) at 500 mkd with or without testosterone propionate Liver: • ↑ Abs wts at 125 (20%) and 500 mkd (53–59%) • ↑ histopath (hepato-cellular hypertrophy, focal/multi-focal hepato-cellular necrosis)
Male Pubertal Assay (OCSPP 890.1500) (Marty et al. 2011b)	*In vivo* pubertal landmarks	0, 50, 200 and 400 mkd			No effects on A endpoints consistent with androgenicity	No effects on: • testicular or epididymal histopath Effects on: • Slight delay in PPS (+1.7 ds) at 400 mkd • Decreased serum testosterone (49 or 85%) • Decreased abs pituitary wts at all dose levels • Decreased abs. seminal vesicle and ventral prostate wts at 200 and 400 mkd • Decreased abs. dorsolateral prostate and LABC at 400 mkd	Increased rel. adrenal wts at 400 mkd (see anti-A effects in prior column)	No effects on T wt or on T4 levels ↑ serum TSH (58%) and altered T histopath at 400 mkd (↑ follicular cell height and ↓ colloid)	• ↓ BW (6.6 or 6.2%) and BWG (8.3 or 8.2%) at 200 or 400 mkd Liver: • ↑Abs/rel wts (12.5–21.3%) at 200 and 26.7–36.5% at 400 mkd along with hypertrophy ↑gamma glutamyl *trans*-peptidase at 400 mkd
OSRI cited: Goetz et al. (2007)	♂ repro dev landmarks following perinatal and PND (GD6 – PND92) exposure to Wistar rats	0,100,500 and 2000 ppm in diet (mkd range for dams during gestation and lactation: 0, 8–19.1, 38.7–93.8 and 141.3–347.3; mkd ranges during postweaning period: 0, 6.1–15.8, 32.9–77.2 and 133.9–280.2)			No effects: • Age at PPS • Serum estradiol or LH levels • Sperm morphology/motility, wt of seminal vesicles, epididymis, hypothalamus, brain or hippocampus • Histopath of androgen-sensitive organs (testis, epididymis, ventral prostate, pituitary) • Total number of implantation sites, live or dead fetuses, live or dead embryos, number of resorptions or postimplantation			No effects that would indicate an anti-T effect: • T wt • T micropath • Serum T3, T4 and TSH	↓ BW (12%) and feed intake in postweaning ♂ at 2000 ppm • ↓ BW at PPS 7.8% at 2000 ppm • ↓ survival rates (82.6%) in litters exposed to 2000 ppm during gestation Liver: • ↑ Rel wt at PND 1, 50 and 92 at 2000 ppm (9–13%) • Centri-lobular hepato-cellular hyper-trophyat PND 92 at 2000 ppm

(*continued*)

Table 11. Continued

Study	Methods	Concentrations tested	E	Anti-E	A	Anti-A	S	Anti-T	Additional findings
					loss% fertility (no. of live fetuses/no. CLs in successful pregnancies) Effects:* • ↑ AGD in ♂ at 2000 ppm • ↓ rel pituitary wt at PND 92 at 500 and 2000 ppm ↑ abs testes wt at PND 1 at 100 and 2000 ppm, and at PND 22 at 500 ppm with no changes at PND 50 or 92 at any dose level • ↑abs and rel ventral prostate wt at PND 92 at 500 ppm (no dose-response) • Impaired insemination with untreated ♀ (reduced ratio of vaginal smears with sperm present) at 2000 ppm • ↓fertility (↓ratio of successful pregnancies per mated, untreated ♀) at 500 and 2000 • ↑serum testosterone at PND 92/99 at 500 and 2000 ppm				
OSRI cited: Tully et al. (2006)	Testis and hepatic biomarkers following a 14-d exposure in ♂ rats	0, 10, 75 and150 mkd for 14 d			• No effect on testis wts • ↑serum testosterone • ↓ sperm motility with no effect on testis histopath			No effects on T histopath	Liver: ↑ Rel wts at 75 and 150 mkd (6–8%) with centrilobular hypertrophy
OSRI cited: Wolf *et l.* (2006)	Markers of T disruption; dietary exposure in ♂ Wistar rats for 4, 30, or 90d	100, 500 and 2000 ppm						No effect on T histology or TSH at any time or exposure • ↓ T4 only (4 d exposure) and T3 only (30 d exposure) (2000 ppm), but • T4, T3 not affected after 90 d	Liver: ↑ UDPGT activity in liver (2000 ppm)

*Goetz et al. (2007): Testis wt determined at PND1, 22, 50 and 92 were inconsistent in myclobutanil treated ♂ with ↑ testes abs wt at PND 1 at 100 and 2000 ppm and at PND 22 only at 500 ppm. ↑ rel testes wt on PND1 at 500 ppm and on PNDs 22 and 50 at 2000 ppm. No wt changes (both rel and abs) were seen on PND 92 at any dose level. Considering the lack of consistency in this study and across other studies, this was not considered evidence of a biologically-significant change. Similarly rel ventral prostate wt was ↑ at 500 ppm, but not at 2000 ppm in F1 ♂ at PND92. Considering the lack of consistency in this study and across other studies, this was not considered evidence of a biologically-significant change. In addition, testosterone levels in F1 ♂ were ↑ on PND92 at 2000 ppm, but not on any other time points or dose levels or in other studies. Considering the lack of consistency, this was not considered evidence of a biologically-significant change.

Table 12. Triadimefon: Part 158 Guideline Toxicology Studies.

Study	Concentrations tested	E	Anti-E	A	Anti-A	Anti-T	Additional findings
Subchronic oral rat (Mohr 1976)	0, 50, 200, 800 and 2000 ppm (0, 2.5, 10, 40 and 100 mkd)	No effects (ovary wt or histopath)		• ↑ rel testes wt (19%) at 2000 ppm • No effects on testes histopath.	No effects on testes histopath	33% ↑ rel T wt, ♀ rats only at 2000 ppm	↑ rel liver wt at ≥50 ppm in ♀ (26–73% at 2000 ppm) and ≥200 ppm in ♂ (27–36%, non-dose dependent)
Subchronic oral dog (Hoffman and Luckhaus 1974)	0, 150, 600 and 2400 ppm (0, 3.75, 15 and 60 mkd)	No effects (ovary or uterine wt or histopath)		No effects (prostate or testes wt, prostate, epididymis, and testes histopath)		No effects (T wt or histopath)	• ↑ rel liver wt (16%) and ↓ BW and food consumption at 2400 ppm • ↑ hepatic enzymes at ≥600 ppm
DNT (Sheets et al. 2008)	0, 100, 300 and 800 ppm (0, 8.0, 23.9 and 71.3 mkd)	No effects (age or BW at VO)		No effects (age or BW at PPS)		No effects (eye opening, brain wt and volume, motor activity, and auditory function (no loss of startle response) at ≤800 ppm	• ↓ maternal BWG (5%) on GD13 and LD0 • ↑ incidence of deviated snout, ↑ startle amplitude on PND60 and number of trials to criterion during retention phase of passive avoidance test (♀ only) • ↓ ♂ pup BWG prior to weaning at 300 ppm • Maternal and offspring NOAEL =300 ppm
Chronic rat (Bomhard and Schilde 1991)	0, 50, 300 and 1800 ppm (approx. 2.5, 15 and 90 mkd)	No effects (ovary or uterine histopath; no organ wt available)		↑ Rel testes wt (5%) at 1800 ppm at 104 wk	No effects (testes or seminal vesicles histopath)	Slight ↑ in T cystic hyperplasia and follicular cell adenomas at 1800 ppm (2 ♀/50 and 4 ♂/50, within historical control range)	• ↑ rel and abs liver wt at 300 ppm (8%) and 1800 ppm (32%) in ♀, and at ↑ rel liver wt (5%) in ♂ at 1800 ppm • ↓ ♂ (7%) and ♀ (13%) BW at 1800 ppm (effects at 104 wk)
Mouse oncogenicity (Bomhard 1986)	0, 50, 300 and 1800 ppm (approx. 7.1, 43.0 and 257 mkd)	No effects (ovary or uterine histopath, no wt available)		No effects (testes wt, or on testes or seminal vesicles histopath)		No effects on T histopath, no wt available)	• ↓ BWG in ♂ and ♀ (≤ 20%) at 1800 ppm • ↑ nonneoplastic (centrilobular hypertrophy) changes and hepatocellular tumors at 1800 ppm • ↑ liver wt (50–60%) at 1800 ppm in ♂ and ♀
Chronic dog (Hoffman and Groning 1978)	0, 100, 330 and 1000 ppm (wk 1–54), or 2000 ppm (wk 55–104) F: 0, 3.5, 12.0, 33.7 (wk 1–54) or 60.4 (wk 55–104) mkd; M: 0, 3.0, 11.4, 34.7 (wk 1–54) or 68.8 (wk 55–104) mkd	No effects (ovary wt or ovary and uterine histopath)		No effects (prostate or testes wt, or on prostate, epididymis and testes histopath)		No effects (T wt or histopath)	↑ rel liver wt and microsomal enzyme induction at 1000 ppm

(*continued*)

Table 12. Continued

Study	Concentrations tested	E	Anti-E	A	Anti-A	Anti-T	Additional findings
Rat developmental toxicity (Nagumo et al. 1981; Unger et al. 1982)	Nagumo (1981): 0, 10, 25, 50 and 100 mkd by gavage Unger, 1982: 0, 10, 30 and 90 mkd	No effects on endpoints that would suggest an E or anti-E effect, including • Gestation duration • Sex ratio • Dev abnormalities suggestive of endocrine effects, e.g., urogenital malformations		No effects on endpoints that would suggest an A or anti-A effect, including: • Gestation duration • Sex ratio • Dev abnormalities suggestive of endocrine effects, e.g., urogenital malformations			• ↑ incidence of supernumerary ribs at 50 and 100 mkd • ↓BWG early in gestation at 50–100 mkd • ↑ motor activity at 25–100 mkd • NOAEL range was 10–30 mkd based on maternal BW
Rabbit developmental toxicity (Clemens 1990)	0, 20, 50 and 120 mkd	No effects on endpoints that would suggest an E or anti-E effect, including: • Gestation duration • Sex ratio Dev abnormalities suggestive of endocrine effects, e.g., urogenital malformations		No effects on endpoints that would suggest an A or anti-A effect, including • Gestation duration • Sex ratio Dev abnormalities suggestive of endocrine effects, e.g., urogenital malformations			• ↓ fetal wt at 120 mkd and minor delays in ossification at 50–120 mkd • ↓ maternal food consumption and reduced BW early in gestation at 120 mkd • Dev NOEL =20 mkd • ↓ BWG at ≥300 ppm • LOAEL =300 ppm
Multigenerational rat study (Loser & Lorke 1979)	0, 50, 300 and 1800 ppm (variable mkd dose dependent on lifestage but average exposure 0, 2.5, 15 and 90 mkd)	No effects (ovary histopath at ≤300 ppm; only F3b pups examined at 0, 50 and 300 ppm; no pups available at 1800 ppm)		No effect on testes or epididymis histopath≤ 300 ppm (only F3b pups examined at 0, 50, and 300 ppm; no pups available at 1800 ppm)	↓ mating by F1 ♂ at 1800 ppm	No effect (T histopath at ≤300 ppm; only F3b pups examined at 0, 50, and 300 ppm; no pups available at 1800 ppm)	
Multigenerational rat study (Eiben 1984)	0, 50 and 1800 ppm (variable mkd dose dependent on lifestage but average exposure 0, 2.5 and 90 mkd)	• ↑ rel and abs ovary wt at 1800 ppm in P only (no effect in F1) • No effects on ovary or uterus histopath at 1800 ppm (only control and 1800 ppm F1 adults examined)	No effects • 1800 ppm dams mated with control ♂ expressed normal fecundity	• No effect on prostate, seminal vesicles, epididymis, or testes histopath at 1800 ppm (only control and 1800 ppm F1 parents examined) • ↑ testes rel wt by 18% at 1800 ppm in the F1 • ↑ serum testosterone by 100% at 1800 ppm (F1 ♂ assessed at 8 mo old and end of study) • No effect on FSH	• ↓ mating (behavior) by F1 ♂ at 1800 ppm with no sperm effects • F0 mating was unaffected	No T wt or histopath available	• ↓ F0 and F1 parental and F1 and F2 pup BW at 1800 ppm • ↑ rel liver wt at 1800 ppm for F0 ♂ and ♀ and F1 ♂ (25%) (↓ for F1 ♀) • For F1–F2 offspring: ↓ litter wt (12%), ↓ viability (50–60%), and ↓ lactation survival (20%) at 1800 ppm • 4 F1 ♂ did not mate at all demonstrated the greatest growth retardation

Table 13. Propiconazole: Part 158 Guideline Toxicology Studies.

Study	Concentrations tested	E Anti-E	A Anti-A	Anti-T	Additional findings§
Subchronic rat (Sachsse et al. 1979a)	0, 240, 1200 and 6000 ppm	No effects on ovary wt or histopath. No effect on mammary or uterine histopath	No effects on testes wts; or on prostate, epididymis, and testes histopath	No effects on T histopath	↑ rel ovary and testis wt at 6000 ppm attributed to large BWG ↓ (≥25%)
Subchronic dog (Sachsse et al. 1979b)	0, 50, 250 and 1250 ppm	No effects on ovary wt or histopath. No effect on mammary or uterine histopath	No effects on testes wts; or on prostate, epididymis, pituitary and testes histopath	No effects on T wt or histopath	
Chronic rat (Hunter et al. 1982a)	0, 100, 500 and 2500 ppm	No effects on ovary wt or histopath. No effects on mammary, uterus, cervix or pituitary histopath*	No effects on testes wts; or on prostate, epididymis, seminal vesicles, pituitary and testes histopath	No effects on T wt or histopath	
Mouse oncogenicity (Hunter et al. 1982b)	0, 100, 500 and 2500 ppm	No effects on ovary wt or histopath. No effects on mammary, uterus or pituitary histopath	No effects on pituitary or testes wts; or on pituitary, prostate, epididymis, and testes histopath	No effects on T wt or histopath	
1 year dog (Johnson & Thompson 1985)	0, 5, 50 and 250 ppm	No effects on ovary or pituitary wt or histopath. No effects on uterus, vagina or mammary histopath	No effects on testes wts; or on prostate, epididymis, and testes histopath. A decrease in rel pituitary wt was seen in 250 ppm ♂, but there were no pituitary histology findings†	No effects on T wt or histopath	
Rat developmental toxicity (Fritz 1984a; Giknis 1987; Mallow et al. 1987)	30 to 300 mkd	No effects on endpoints that would suggest an E or anti-E effect, including • Gestation duration • Sex ratio • Dev abnormalities suggestive of endocrine effects (e.g., urogenital malformations)	No effects on endpoints that would suggest an A or anti-A effect, including • Gestation duration • Sex ratio • Dev abnormalities suggestive of endocrine effects (e.g., urogenital malformations)		General maternal toxicity (including ↓ BW) at 300 mkd
Rabbit developmental toxicity (Fritz 1984b; Raab 1987)	Fritz (1984b): 0, 30, 90 and 180 mkd Raab (1987): 0, 100, 250 and 400 mkd	No effects that would indicate an E or anti-E effect Slightly ↑ abortion/early parturition at high dose in one study (Raab 1987). No similar findings in second rabbit study (Fritz 1984b)‡	No effects on endpoints that would suggest an A or anti-A effect, including: • Gestation duration • Sex ratio • Developmental abnormalities suggestive of endocrine effects (e.g., urogenital malformations)		Raab (1987): ↓ BW and food consumption at 250 and 400 mkd and clinical signs at 400 mkd
Multigeneration Reproduction Study (Borders & Salamon 1985)	0, 100, 500 and 2500 ppm (0, 8.1, 41.4 and 200.4 mkd – F1 ♀ at wk 10)	No effects on endpoints that would suggest an E or anti-E effect, including • Repro • Sex ratio • Ovary wt • Histology of ovary, uterus, vagina and pituitary	No effects on endpoints that would suggest an A or anti-A effect, including: • Repro • Sex ratio • Testis wt¶, Histology of prostate, seminal vesicles, pituitary and testes with epididymides	No effect on T histopath	At 2500 ppm, ↓ number of pups delivered and surviving to PND4 in F2a litters; ↓ number of pups surviving PND 7-21in F2b litters = offspring toxicity. No evidence of a repro or endocrine-mediated effect

NT, not tested.

*Chronic rat study: US EPA considered incidence of uterus luminal dilatation at 2500 ppm (17/65 versus 4/58 control) to be treatment related, but subsequent examination of the report indicated that this was a normal age-related finding in 2-year old rats, that was recorded under a variety of slightly different pathology descriptors in the control group.

†One-year dog study: in the absence of any micropathology findings or effects on abs organ wt, lower rel pituitary wt (♂ dogs, 250 ppm) was not considered a treatment-related effect.

‡Rabbit developmental toxicity study (Raab 1987): slight ↑ abortion/early parturition at 400 mkd was seen in the presence of significant maternal toxicity (BW, food consumption, clinical signs) and is considered a result of general toxicity.

¶Multigeneration repro study: abs testes wts were lower than control at 2500 ppm in F2A and F2B offspring, but not adverse since no effect on rel wts and no micropathology changes. Absolute testis wts in the F1A and F1B offspring were unaffected.

§In all rat and mouse studies, effects on ↓ BWG and liver wt/liver histopathology were observed that defined the LOAEL and NOAEL values. Liver histopathology in rodents included hepatocyte hypertrophy, vacuolation, necrosis (mice only) and adenomas/carcinomas (mice only; 2500 ppm). NOAEL values for these effects were at 100 ppm (10 mkd) in mice and at 500 ppm (18 mkd) in rats (EPA 2014b).

Table 14. Myclobutanil: Part 158 Guideline Toxicology Studies.

Study	Concentrations tested	E	Anti-E	A	Anti-A	Anti-S	Anti-T	Additional findings – systemic toxicity
Subchronic Mice, 1986 (Goldman et al. 1986a)	Dose levels: 0, 3, 10, 30, 100, 300, 1000, 3000 and 10000 ppm For ♂, 0.40, 1.54, 4.79, 14.1, 42.7, 132, 542 and 2035 mkd and for ♀ 0.62, 2.11, 6.94, 22.9, 65.5, 232, 710 and 2027 mkd	• No effects	• Immature uterus and absence of corpora lutea in the ovaries (no ovulation) at 10000 ppm	• Increased rel testis wt (15%) at 10000 ppm secondary to terminal BW decreases	• No effects	• Adrenals:↑ rel wts at 10,000 ppm secondary to ↓ BW and ↑ eosinophilia and/or hypertrophy of zona fasciculata at ≥1000 ppm	No effects	Systemic toxicity at 3000 ppm (exceeded the MTD at 10,000 ppm) • Severe BW ↓ (21%) at 10,000 ppm with ↓ feed consumption; also ↓ BW in ♂ at 3000 ppm (7%) • Lymphoid necrosis in spleens at ≥3000 ppm and thymus and mesenteric lymph nodes at 10,000 ppm Liver: • ↑ Rel wt at ≥1000 ppm (35–151% in ♂; 18–141% in ♀) • Hepatocellular hypertrophy, vacuolation, necrosis and necrotic hepatitis at ≥1000 ppm
Subchronic Rats, 1984 (O'Hara & DiDonato 1984)	0, 10, 30, 100, 300, 1000, 3000, 10,000 and 30,000 ppm from wk 5 onward (dose levels were ↑) (mkd ranges: 0, 0.52–0.67, 1.6–2.0, 5.2–6.8 15–20, 51.5–65.8 158–195, 585–665 and 1730–1811 mkd)	• No effects	• No effects	• No effects	• No effects	• Vacuolation of adrenal cortex in ♂ at (1000), 3000 and 10,000 ppm and in ♀ at 3000 and 10,000 ppm	• ↑ Rel T wt in ♂ of 3000 (21%) and 10,000 (38%) ppm and in ♀ of the 10000-ppm (19%) group • ↑ small follicle number in T of ♂ at 3000 ppm and 10000 ppm	Systemic toxicity at 10,000 ppm (Exceeded the MTD at 30,000 ppm-): • All were dead at 30000 ppm, including ♂ by TD63 and ♀ by TD49 (exceeded the MTD). ↓ BW at 10000 ppm indicative of systemic toxicity (30% in ♂ and 12% in ♀) Liver: • ↑ Rel liver wts at ≥3000 ppm (38–128% in ♂ and 33–104% in ♀), • Hypertrophy and single cell necrosis at ≥3000 ppm • ↑ BUN, cholesterol and globulin at ≥3000 ppm
Subchronic rats, 1987 (Shimizu 1987)	0, 100, 300 and 3000 ppm; average mkd: 0, 6.2, 18.8 and 191.5 in ♂ and 6.9, 19.6 and 224.9 in ♀; 10/sex/dose	• No effects	• ↑ Abs./rel. ovarian wt only at mid dose 300 ppm (no dose-response) • No histopath changes observed	• No effects	• No effects on pituitary wt • No effects on repro/ASG histopath • ↓ Abs./rel. prostate wt at 100 and 3000 ppm (not dose-related)	• ↓ Abs. adrenal wts in ♂ at 3000 ppm; ↓ rel. adrenal wts ≥100 ppm in ♂ • Slight vacuolation of cortical cells, slight atrophy of the zona	• No effects	Systemic toxicity at 3000 ppm (Exceeded MTD in ♀-: 17% ↓ in BWG) ↓9% in ♂ BWG

(*continued*)

Table 14. Continued

Study	Concentrations tested	E	Anti-E	A	Anti-A	Anti-S	Anti-T	Additional findings – systemic toxicity
					• Atrophy of seminiferous tubule, giant cell-like change and absence of sperm cells in epididymis in 1 high-dose ♂ • These effects are not dose dependent and are considered spontaneous	fasciculata, fine vacuolation of zona glomerulosa at high dose		Liver: • ↑ Abs (9–17%)/rel (19–23%) liver wts at 3000 ppm in males and females Slight or moderate hepatocellular hypertrophy at 3000 ppm with focal necrosis • ↓ Triglycerides and glucose in ♂; ↓ bilirubin in both sexes at 3000 ppm Kidney: • ↑ Abs/rel kidney wts in ♂ at 3000 ppm • Slight vacuolar degeneration of renal tubule epithelium, dilated renal pelvis at 3000 ppm
Subchronic dogs, 1984 (4/sex/group) (McLaughlin & DiDonato 1984)	Dose levels= 0, 10, 200, 800 and 1600 ppm 0, 0.34–0.42, 7.26–7.88, 29.13–32.43 and 56.8–57.97 mkd	• No effects	• ↑ rel ovary wts ≥800 ppm attributed to estrus; not treatment related • No histopath changes observed.	No effects	No effects	• ↑ rel adrenal wts in ♀ at 1600 ppm	No effects	Liver: • ↑ Rel wts at ≥800 ppm (24–41% in ♂ and 12–31% in ♀) • ↑ serum alkaline phosphatase • hypertrophy in both sexes at >200 ppm
Chronic rat, 1986 (110/sex/dose) (Shellenberger & Billups 1986)	Dose levels: 0, 50, 200 and 800 ppm 0, 2.49–3.23, 9.84–12.86 and 39.21–52.34 mkd	• No effects	• ↑ abs and rel ovary wts at 800 ppm (not statistically significant and at 12 mos. only) • No histopath changes observed	• No effects	• ↑ incidence of testicular atrophy (degeneration of seminiferous tubules) at ≥200 ppm) • ↓ testis wt at ≥200 ppm	• No effects	No effects	• ↓ BW (2–9%) in 800 ppm ♂ and ♀ at some intervals up to 96 wks Liver: ↑ rel wts (9–15%) at 800 ppm in both sexes
Chronic rat, 1993 (Wolfe 1993)	Dose levels =0 and 2500 ppm 0 and 106–136 mkd	No effects	No effects	• No effects	Aspermato-genesis, hypospermia and cellular debris in the epididymides at 2500 ppm	No effects	No effects	Liver: Hypertrophy and vacuolation at 2500 ppm
Mouse oncogenicity, 1986 (Goldman and Harris 1986)	Dose levels =0, 20, 100 and 500 ppm 0, 2.7–3.2, 13.7–16.5 and 70.2–85.2 mkd	No effects	No effects	No effects	No effects	No effects	No effects	• Slight/individual necrosis and foci of altered hepatocytes and multifocal hepatocellular vacuolation in ♂ and ♀ at 500 ppm
Mouse oncogenicity, 1993 (targeted study) (Andersen et al. 1993)	Dose levels =0 and 2000 ppm 0 and 393.5 mkd (only ♀)	No effects	No effects	No effects	No effects	• Adrenal cortex hypertrophy of zona fasciculata at 2000 ppm	No effects	• ↓ BW (12%) and food consumption Liver: ↑ Rel wt (33%), ↑ hepatocellular hypertrophy and vacuolation; single cell

(*continued*)

Table 14. Continued

Study	Concentrations tested	E	Anti-E	A	Anti-A	Anti-S	Anti-T	Additional findings – systemic toxicity
								necrosis and pigmented Kupfer cells
1 year dog, 1986 (6/sex/group) (Goldman et al. 1986b)	Dose levels =0, 10, 100, 400 and 1600 ppm 0, 0.34–0.40, 3.09–3.83, 14.28–15.68 and 54.22–58.20 mkd	No effects	No effects	No effects	No effects	No effects	No effects	Liver: • ↑ Abs/Rel wts (44/52%) and hypertrophy in ♂ and ♀ at ≥400 ppm ↑ serum alkaline phosphatase and ballooned heptocytes in ♂ and ♀ at 1600 ppm
Rat developmental toxicity, 1984, 2005 (Costlow & Kane 1984a)	0, 31.3, 93.8, 313 and 469 mkd	No developmental effects that are suggestive of an E or anti-E effect		No developmental effects that are suggestive of an E or anti-E effect		No effects	No effects	• General maternal toxicity (bloody urine, scant feces, salivation, staining of urogenital area) at ≥312.6 mkd • ↓viability index at >93.8 mkd
Rabbit developmental toxicity, 1984 (Costlow & Kane 1984b)	0, 20, 60 and 200 mkd	• ↑ abortions and resorptions at 200 mkd	No effects suggestive of an anti-E effect	No developmental effects that are suggestive of an E or anti-E effect		No effects	No effects	• ↓ BW (3%) at 200 mkd during gestation; clinical signs • Embryotoxic at 200 mkd-decreased viability index and ↓ fetal BW
Rat two-generation reproductive toxicity study, 1985 (Costlow & Harris 1985) (parental animals mated twice in each generation) – 25/sex/generation	0, 50, 200 and 1000 ppm (0, 4, 16 and 80 mkd)	• At 1000 ppm • ↓ no. ♀ delivering litters in 2 of 4 matings • Slight ↓ P2 ♀ mated (sperm +) • Slight ↑ time to mating (2 animals) • ↑ still-born pups (4 of 4 matings)		No effects	At 1000 ppm • Testicular atrophy (P2 only) • Decreased amounts of spermatozoa (P2 only) • Necrotic spermatocytes/spermatids in epididymal tubules (P2 only) • Prostate atrophy (P2 only)	Adrenals were not examined	NA	Systemic toxicity at 1000 ppm • ↓BW (9%) at 1000 ppm in P2 ♂ prior to mating (due to lower weanling wts); transient decrease in P1 ♂ from wks 1–2 pre-mating • ↓ BWG in pups during lactation at 1000 ppm (increasing effect until weaning) • Greater effects in P2 attributed to higher dosages during lactation and post-weaning periods • ↓ Litter size in 1 of 4 matings Liver: ↑ Rel wts (4–13%), hypertrophy, and vacuolization in P1 and P2 adults at ≥200 ppm (only adaptive changes in ♀)

Table 15. Triadimefon: Bioactivity concordance in mammalian toxicology assays across three lines of evidence.

Endocrine activity	HTS	Part 158 guideline studies	EDSP Tier 1 + published OSRI	Concordance
S	*In vitro* inhib of aromatase at concentrations that may result in cytotoxicity		*In vitro* inhib of aromatase	*Concordant*. The *in vitro* results of HTS and Tier 1 OSRI appear concordant, suggesting *in vitro* inhib of aromatase and no direct interaction with ER or AR
E Receptor binding	*Negative*		*Negative*	
E Receptor transactivation	AUC model score 0.0095 (*Negative*)		*In vitro* transactivation at concentrations that may result in cytotoxicity	
A Receptor binding	AUC model score 0 (*Negative*)		*Negative*	
A Receptor transactivation	*Negative*		*Negative*. A published AR transactivation assay (Kojima et al. 2004) was negative for agonist and antagonist interactions	
T receptor activity	*Negative*			
In vitro anti-T activity	27/77 hepatic catabolism assays were *positive* for triadimefon. Assay data suggest a potential activation of hepatic nuclear receptors that could result in ↑ metabolism and clearance of T hormones *in vivo*. However, only two expression assays (CLZD_UGT1A1_24 and CLZD_SULT2A1_48) are linked directly to T hormone catabolism.			There are no *in vitro* anti-T studies available to compare to the HTS dataset HTS results showing ↑ UDPGT and SULT expression are concordant with effects observed *in vivo* in rat and mouse liver (including toxicogenomic data) and T effects.
In vivo E activity		↑ rel and abs ovary wt at 1800 ppm in P generation only Multigeneration study (Eiben 1984)	↑ rel ovary wt at 1800 ppm (Rockett et al. 2006).	*Concordant*. *In vivo* there are few, minor findings, including ↑ ovary wt at 1800 ppm in both guideline and Tier 1+ OSRI studies. These findings do not support the hypothesis that triadimefon exerts E-related effects; rather, these may be attributable to S effects
In vivo anti-E activity		*Negative* – across 11 studies in rats, mice, rabbits and dogs	Delayed VO, abnormal estrous cyclicity at 1800 ppm (Rockett et al. 2006)	*In vivo*, some suggestion of anti-E comes from OSRI. However, these few effects may be due to nonspecific toxicity or effects on S
In vivo A activity		↑ serum testosterone and rel testis wt in F1 at 1800 ppm (Multigeneration study, Eiben 1984)	• ↑ serum testosterone at 500 (Goetz et al. 2007) and 1800 ppm (Goetz et al. 2007, 2009) • ↑ abs/rel testes wt at mid-dose (500 ppm) (high dose data unavailable) (Goetz et al. 2007) • ↑ AGD at 1800 ppm (Goetz et al. 2007)	*Concordant*. ↑serum testosterone *in vivo* may be consistent with a perturbation of S pathways
In vivo anti-A activity		↓ F1 ♂ mating behavior at 1800 ppm Multigeneration studies (Eiben 1984, Loser & Lorke 1979)	• ↓ F1 ♂ mating behavior; delayed PPS at 1800 ppm (Goetz et al. 2007) • ↓ fecundity in FSTRA	*Concordant*. ↓ ♂ mating behavior in rodents at 1800 ppm was evident for both guideline and EDSP/OSRI studies
In vivo T activity		*Negative* – across 11 studies in rats, mice, rabbits and dogs	*Negative* in OSRI used in place of pubertal assays and other short-term studies	*Concordant: Negative*
In vivo anti-T activity		Slight ↑ T cystic hyperplasia and follicular cell adenomas at 1800 ppm (Chronic rat study, Bomhard 1991)	• ↓ serum T4 at 1800 ppm (Wolf et al. 2006; Goetz et al. 2007) • Mild ↑ follicular cell hypertrophy at 1800 ppm (Wolf et al. 2006; Rockett et al. 2006) • ↑ T colloid depletion; ↓ serum T3 and TSH at 1800 ppm, depending on exposure duration; ↑ hepatic UDPGT activity at 30 and 90 d treatment; ↑ cell proliferation in the T at 30 d (1800 ppm) (Wolf et al. 2006).	*Concordant*. Both dose-dependent and duration-dependent effects on the rodent T are apparent, with effects on T hormones at 1800 ppm and changes in T histopath at the same dose with long-term exposure. Findings are consistent with increased hepatic catabolism of thyroid hormones, as suggested by the *in vitro* anti-T HTS results

Table 16. Propiconazole: Bioactivity concordance in mammalian toxicology assays across three lines of evidence.

Endocrine activity	HTS	Part 158 guideline studies	EDSP Tier 1 + published OSRI	Concordance
In vitro Steroidogenesis (S)	*In vitro* inhib of aromatase at 2 uM or 24 uM		*In vitro* inhib of aromatase and altered S	*Concordant*. Both suggest inhib of S at possibly cytotoxic concentrations (>1–24 uM)
E Receptor binding	*Negative*		*Negative*. Demonstrated disruption of the binding assay at high conc. (0.6–1.2 mM)	*Concordant = negative*
E Receptor transactivation	AUC model scores 0 (E), 0 (anti-E) Few *positive* results (3/16 for E; 2/14 for anti-E) at high conc. (most ≥25 μM)		*In vitro* transactivation either activated (25 μM) or inhibited (52 μM) at very high concentrations	*Concordant*. Few positive results for interaction with ER at high concentrations (most >25 μM), likely at cytotoxic concentrations
A Receptor binding A Receptor transactivation	AUC model score 0 (*Negative*) for A; AUC model score 0.11 (weak) for anti-A; with 5/8 assays positive in 13–70 μM range		Binding and reporter assays: generally negative for AR activation; weak positive for anti-A *in vitro* effects (4–96 μM range)	*Concordant*. Generally negative for A and weak positive *in vitro* for anti-A effects (4–96 μM) *in vitro*, likely at cytotoxic concentrations
T receptor activity	*Negative* (activation or inhib)		Not tested (not a part of EDSP Tier 1 tests)	
In vitro anti-T activity	27/76 hepatic catabolism assays were positive, but most were non-specific CYP induction. Only 1 conjugation assay positive at high concentration (SULT2A1 – 48 h); no effect on UDPGT1A1 activity		Not tested (not a part of EDSP Tier 1 tests)	See *in vivo* assays related to T.
In vivo E activity		*Negative* – across 11 studies in rats, mice, rabbits and dogs	*Negative* – across *in vivo* uterotrophic, ♀ pubertal, and other studies	*Concordant*. Negative in the *in vivo* studies
In vivo anti-E activity		*Negative* – across 11 studies in rats, mice, rabbits and dogs	*Negative* in mammalian studies (♀ pubertal + OSRI)	*Concordant*. Negative in mammalian studies
In vivo A activity		*Negative* – across 11 studies in rats, mice, rabbits and dogs.	*Negative* in all mammalian studies, except: ↑ AGD at 2500 ppm in F1 ♂ (Goetz et al. 2007); no effect on AGD in ♀ or in several other similar studies (Taxvig et al. 2008; Rockett, 2006)	*Concordant*. Negative except for one non-reproducible finding
In vivo anti-A activity		*Negative* – across 11 studies in rats, mice, rabbits and dogs	*Negative* in mammalian studies (♂ pubertal, Hershberger and OSRI)	*Concordant*. Negative in mammalian studies
In vivo steroidogenesis		*Negative* – across 11 studies in rats, mice, rabbits and dogs	*Negative* in mammalian studies (♂ pubertal, ♀ pubertal, Hershberger + OSRI)	*Concordant*. Negative in mammalian studies
In vivo thyroid activity		*Negative* – across 11 studies in rats, mice, rabbits and dogs	*Negative* – in amphibian, ♂ pubertal, ♀ pubertal, Hershberger + OSRI studies	*Concordant – Negative*.
In vivo anti-thyroid activity		*Negative* – across 11 studies in rats, mice, rabbits and dogs	*Negative* – in amphibian, ♂ pubertal, ♀ pubertal, Hershberger + OSRI studies. One OSRI (Wolf et al. 2006): ↓ serum T4 on days 4 and 30; ↑ UDPGT activity in liver (2500 ppm), but T4, T3 and TSH normal by d 90 in rats and no T micropathology seen	*Concordant – Negative* One finding (lower T4 in rats at 2500 ppm) could be secondary to ↑ UDPGT activity (1-naphthol as substrate) in liver of rats; effect was transient with no functional consequence

Table 17. Myclobutanil: Bioactivity concordance across three lines of evidence.

Endocrine activity	HTS	Guideline studies	Tier 1 + OSRI package	Concordance
In vitro Steroidogenesis (S)	*In vitro* inhib of aromatase with one AC_{50} value greater than the cytotoxicity caution flag		*In vitro* inhib of aromatase and S	*Concordant*. The *in vitro* results of HTS, OSRI and Tier 1 appear concordant, suggesting *in vitro* inhib of aromatase and no direct interaction with ER or AR
E Receptor binding	*Negative*		*Negative*	
E Receptor transactivation	AUC model score 0 (*Negative*)		*Negative*	
A Receptor binding	AUC model score 0 (*Negative*)		*Negative*	
A Receptor transactivation	*Negative*			
T receptor activity	*Negative*			
In vitro anti-T activity	19/79 hepatic catabolism assays were *positive*. Assay data suggests a potential activation of hepatic nuclear receptors that could result in ↑metabolism and clearance of T hormones *in vivo*			See *in vivo* assays related to T
In vivo E activity		• *Negative* ((sub-chronic and chronic studies) • ↑ rel ovary wts considered spurious and not treatment related with either no dose response or occurrence concomitant with systemic toxicity)	• *Negative* (across *in vivo* uterotrophic and ♀ Pubertal Tier 1 assays) • ↑ rel ovary wt at 2000 ppm (Rockett et al. 2006)	• *Concordant. Negative*. No evidence of estrogen activity exerted by myclobutanil across all the lines of evidence-Part 158, Tier 1 and or OSRI
In vivo anti-E activity		• *Negative* (multigen, effects seen attributed to maternal toxicity)	• *Negative* (Female Pubertal Tier 1 Assay) No effects on age at VO, estrous cycling, estradiol levels, ovarian or uterine wts or histopath • **OSRI:** ↑ AGD and delayed VO at 2000 ppm (Rockett et al. 2006)	• *In vivo*, some indictorss of anti-E effects from OSRI. However, these effects are likely due to nonspecific toxicity and/or effects on S. No indicators of direct anti-E potential
In vivo A activity		*Negative*	• *Negative* (Hershberger and male pubertal) • **OSRI:** No delay in PPS ↑ AGD, testesterone levels, ↑ testes wts and rel ventral prostate wt; reduced litter survival, impaired insemination and fertility (at 2000 ppm with toxicity) (Goetz et al. 2007)	• *Concordant –Negative* • Androgenic effects seen in Goetz et al. (2007) was not evident in any other studies
In vivo anti-A activity		• Effects on testes, prostate and epididymes (atrophy, ↓ amounts of spermatozoa); ↓ litter size, ↑ stillborn pups & no. ♀ deliveringat 88 mkd (2-gen Rat Study) • ↑ incidence of testicular atrophy at ≥9.84 mkd (Chronic Rat study)	• Delayed PPS (+ 1.7 d) only at 400 mkd (no change in bwt at PPS); ↓ testosterone levels and AST wts at 200 and 400 mkd (↑ abs and rel. liver wt) (male pubertal assay) • Reduced mating of F1 ♂ at 2000 ppm coincident with ♂ wt decreases (Goetz et al. 2007)	*Concordant* Anti-A effects were evident for part 158/EDSP and OSRI • Full database suggests altered steroidogenesis (via aromatase inhibition) accompanied by liver changes at these high-dose levels in mammals *in vivo* • No indications of a direct anti-A effect
In vivo T activity		*Negative- across all Part 158 studies*	*Negative-in amphibian,* pubertal assays and OSRI	*Concordant.: Negative*
In vivo anti-T activity		↑ rel T wt and ↑ small follicles at 3000–10000 ppm; (51.5–195 mkd) (only seen in sub-chronic rat, not seen consistently)	↑ TSH and altered histopath seen only in ♂ pubertal assay (Tier 1) only at higher dose level (400 mkd); Changes in thyroid hormones seen in only one study but no thyroid histology (Wolf et al. 2006) [↓ serum T4 only on d 4 and T3 only on d 30 (2000 ppm)]	Effects on T are not evident consistently across multiple studies. Weak effect that may be dose- and time-dependent. Effects likely secondary to ↑UDPGT hepatic enzyme induction and T hormone clearance

to altered steroidogenesis pathways and/or systemic toxicity (including a likely contribution of neurotoxicity via inhibition of dopamine reuptake), along with effects on hepatic metabolism in multiple species that may have increased thyroid hormone clearance and may be associated with a slightly increased incidence of hyperplasia and follicular cell adenomas in the thyroid of rats following chronic exposure (Table 12). Neither effects on male mating behavior nor potential effects on the thyroid in rats appeared to be mediated directly; there were no effects observed on sperm function or histopathology in endocrine-relevant tissues in a multi-generation study (Eiben 1984), and triadimefon effects on the thyroid were concomitant with liver effects and required high-dose (1800 ppm), chronic exposure in rats (Bomhard & Schilde 1991). Thus, a dietary exposure of 1800 ppm or doses of approximately 100 mkd triadimefon (dependent on size and age of the animal) appeared to correspond to a dose that may produce systemic toxicity, as well as some changes in rat mating behavior (and resultant fecundity) and perturbations in liver-mediated thyroid hormone homeostasis in rats.

Propiconazole

Guideline mammalian toxicology (40 CFR Part 158) studies in various mammalian species with propiconazole showed no consistent evidence of effects on estrogen, androgen, steroidogenesis or thyroid systems (Table 13). Sub-chronic and chronic toxicity studies in rat, mice and dogs did not show any effects on weights of endocrine-sensitive organs or histological changes, including testes, ovaries and thyroid. In mice and rats, effects in the liver were the most consistent target-organ toxicity, with an increase in hepatocellular adenomas and carcinomas in male mice at 2500 ppm. Any effects reported on offspring viability and BW (Borders & Salamon 1985) in the two-generation rat study appear to be evidence of offspring toxicity rather than a primary endocrine-mediated effect. Thus, dietary exposure of 2500 ppm or doses of approximately 200 mkd propiconazole (dependent on size and age of the animal) appear to correspond to systemic and liver toxicity, with any effects on offspring survival in the multi-generation reproduction study secondary to these primary effects.

Myclobutanil

A review of the 40 CFR Part 158 guideline toxicology data indicates that myclobutanil can affect male reproductive endpoints in rats at high-dose levels (Table 14). In studies with a high dose that did not exceed the maximum tolerated dose (MTD), male rats exhibited decreased prostate or testis weights and alterations in testicular histopathology and spermatogenesis. Although aromatase inhibitors have been shown to transiently alter spermatogenesis in male rats (Nunez et al. 1996; Gerardin & Pereira 2002; Pouliot et al. 2013), myclobutanil did not demonstrate the expected pattern of additional effects typically seen with potent aromatase inhibitors (Moudgal et al. 1996). In particular, ovarian histology and estrous cycles are thought to be very sensitive to aromatase inhibitors but were not affected by myclobutanil. Further, aromatase inhibitors have been associated with multiple developmental effects in rats, including decreased survival, fetal hematomata, and skeletal anomalies (Tamada et al. 2004; Tiboni et al. 2008, 2009), but these effects were not observed in the rat (or rabbit) developmental studies. Thus, myclobutanil did not display a consistent pattern of effects typical for aromatase inhibition, suggesting that *in vivo* this triazole may have more than one MOA for toxicity, including altered liver-mediated steroid hormone metabolism at high-dose levels. Thyroid effects (increased thyroid weight and/or histopathology) in the rat sub-chronic study appear to have been secondary to increased liver weights and hepatocellular hypertrophy; any observed thyroid effects appeared to be secondary to increased hepatic catabolism of T4 at high doses of myclobutanil. Overall, a dietary exposure of 200 ppm or doses of approximately 9.8–12.8 mkd myclobutanil appeared to correspond to a dose that may affect male reproductive endpoints via aromatase inhibition and/or liver toxicity, resulting in altered liver hormone homeostasis in rats.

Bioactivity concordance across three lines of evidence: HTS results, EDSP Tier 1 results and 40 CFR Part 158 guideline studies plus other OSRI

The purpose of this concordance assessment is to identify, on a qualitative basis, whether HTS results and 40 CFR Part 158 guideline studies, considered together in a weight-of-evidence approach, would have provided the needed information for EDSP screening. To evaluate this question, the degree of concordance across the three difference lines of evidence for each triazole fungicide is examined for each of the different endocrine pathways (Tables 15–17).

Triadimefon

The HTS data, EDSP Tier 1 assays and published OSRI and 40 CFR Part 158 guideline toxicology tests were generally concordant for *in vitro* and *in vivo* endocrine activity hypotheses, when data were available from one or more lines of evidence for comparison (Table 15). The negative ToxCast ER and AR AUC model scores for triadimefon demonstrate bioactivity concordance with the *in vitro* screens and Hershberger results of the EDSP Tier 1 evaluation and the published OSRI studies that were similar to male and female pubertal studies (Rockett et al. 2006; Goetz et al. 2007). Triadimefon was classified as "non-interacting" in the guideline AR and ER binding assays (Willoughby 2012a, b). Overall, it appears clear that any potential endocrine activity is associated with a high *in vivo* dose in rats of 1800 ppm. For triadimefon, the HTS results do not suggest estrogenic/anti-estrogenic or androgenic/anti-androgenic potential via a receptor interaction. Rather, these HTS data forecast a potential inhibition of aromatase activity using two separate assay platforms – cell-free and cell-based aromatase inhibition assays – but at concentrations that may exceed a cytotoxicity threshold. These data suggest a potential interference with steroidogenesis with low potency and at concentrations that might cause non-specific assay responses. Concordant with the Goetz et al. (2009) steroidogenesis assay results, triadimefon decreased testosterone production levels in a testis organ culture from the same study, with

simultaneous increases in progesterone production, suggestive of CYP17A1 inhibition (Goetz et al. 2009). In contrast, high-dose (1800 ppm) triadimefon exposure for 30 d increased serum testosterone levels *in vivo* in male rats (Goetz et al. 2009) and also increased serum testosterone in a perinatal exposure study in male rats (Goetz et al. 2007), but it did not change estradiol levels in these studies or in a study similar to a female pubertal assay (Rockett et al. 2006) (Table 9). For triadimefon, although it inhibited aromatase *in vitro*, the direction of effects on testosterone production was not consistent between *in vitro* and *in vivo* assays, possibly because any *in vivo* effects were obscured by systemic toxicity.

Effects on reproductive endpoints with triadimefon may have been mediated at least in part by systemic toxicity and the known effects of triadimefon on neurobehavior at these doses. Triadimefon exposure (1800 ppm) increased relative ovary weight and delayed VO in a published study similar to a female pubertal assay (Rockett et al. 2006) and delayed PPS, increased anogenital distance (AGD), increased testis weight and increased serum testosterone in the absence of any histopathological changes in the testes or effects on sperm morphology and motility in a published study similar to male pubertal assay (Goetz et al. 2007) (Table 9). Effects observed at 1800 ppm in the rat were coincident with systemic toxicity markers, including decreased BW and litter survival (Rockett et al. 2006; Goetz et al. 2007). Lower reproductive success for F1 male rats was also observed with triadimefon exposure at 1800 ppm in a study similar to a male pubertal (Goetz et al. 2007) and in two 2-generation reproduction studies (Loser & Lorke 1979; Eiben 1984). Goetz et al. (2007) observed that mating 1800 ppm-treated males with control females still resulted in depressed fertility rates. These effects, however, were demonstrated to be isolated to male reproductive behavior, as when untreated male F1 rats were cross-mated with 1800 ppm-treated F1 females, the insemination and fertilization rates were not different from control (Eiben 1984).

Additional published reports (Crofton 1996; Walker & Mailman 1996) have demonstrated that triadimefon inhibits dopamine transporter-mediated uptake, which may contribute to decreased pup BW and lactation index (survival) via diminished milk production and altered maternal behavior (Richardson et al. 1984; Freeman et al. 2000; Ben-Jonathan & Hnasko 2001; Price & Bridges 2014), as well as contributing to altered male mating behavior and subsequent reduced fecundity in rat multi-generation studies. Thus, it appears that the HTS assay data, EDSP Tier 1 data, and guideline toxicology data were concordant, i.e., there was evidence for potential endocrine activity resultant to effects on steroidogenesis, which *in vivo* occurred at doses that also resulted in systemic toxicity and possibly neurotoxicity.

Concordance is also apparent for potential bioactivity related to thyroid signaling. All three lines of evidence suggest a potential activation of hepatic nuclear receptors, including CAR and/or PXR, which could result in increased metabolism and clearance of thyroid hormones *in vivo* (Goetz & Dix 2009). HTS assays also indicated that triadimefon might upregulate UGT1A1 and SULT2A1 expression in primary hepatocytes, enzymes known to catabolize T4 (thyroxine) and T3 (triiodothyronine) (Butt & Stapleton 2013; Tong et al. 2007; Richardson et al. 2014). Chronic triadimefon administration in rats increased liver weight, induced microsomal enzymes and caused centrilobular hypertrophy consistent with upregulated Phase I (and II) metabolism (Bomhard & Schilde 1991) (Table 12). This chronic, high-dose (1800 ppm) exposure to rats also resulted in a slight increase in the incidence of thyroid follicular cell adenomas and cystic hyperplasia (Bomhard & Schilde 1991), consistent with decreased thyroid hormones and increased thyroid stimulating hormone (TSH) signaling. Long-term exposures are absent from the EDSP Tier 1 battery, but a study similar to a male pubertal assay did not produce thyroid histological alterations despite decreased blood T4 levels at 1800 ppm (Goetz et al. 2007). Exposure of male rats to 1800 ppm triadimefon decreased T4 and T3 concentrations after 4 and 30 d, decreased TSH at 4 d of exposure, and increased follicular cell hypertrophy, colloid depletion and cell proliferation in the thyroid at 30 d of exposure; however, no thyroid effects were observed at 90 d of exposure (Wolf et al. 2006). In this same study, 1800 ppm triadimefon exposure induced hepatic UDP-glucuronosyltransferase (UDPGT) activity (with 1-naphthol as substrate) and increased hepatocellular hypertrophy. In a study similar to a male pubertal assay, relative liver weights increased, BW decreased and pup survival was reduced at this same dose (1800 ppm) (Goetz et al. 2007). In aggregate, the data suggest a primary effect on hepatic catabolism that may correlate with decreased serum thyroid hormones in rodents *in vivo*. Under chronic, high-dose exposure conditions, this may lead to increased TSH that can culminate in aberrant thyroid histopathology in rodents, a tumor pathway that is not considered human relevant (McClain 1995; IARC 1999; McClain & Rice 1999; Dellarco et al. 2006; Rouquie et al. 2014).

Propiconazole

Comparison of the three lines of evidence for propiconazole suggests *in vitro* inhibition of aromatase that is largely without endocrine-related apical effects in mammalian *in vivo* studies (Table 16). Thus, the HTS assay data for aromatase inhibition is concordant with *in vitro* assay data used to satisfy the EDSP Tier 1 data requirements and serves as a conservative screen, because related effects were not observed *in vivo*. For propiconazole, the AUC ER and AR model scores and HTS assay results were also concordant with the *in vitro* screens that were part of the EDSP Tier 1 testing and published OSRI. Propiconazole did not interact with the ER, and ER transactivation assays (HTS screens and EDSP Tier 1 results) demonstrated inconsistent results at high concentrations that were likely cytotoxic. The AUC AR model score for androgenic effects was 0, and this correlated with the lack of AR activation in the OSRI that satisfied the EDSP Tier 1 data requirements. An AUC score of 0.11 for anti-androgenic effects with propiconazole (indicating a weak positive) was concordant with OSRI study results, including four published *in vitro* studies (Table 10) that displayed evidence of antagonism of the AR at relatively high concentrations (3–96 μM). In contrast, *in vivo* studies that were part of the EDSP Tier 1 testing plus OSRI and the guideline 40 CFR Part 158 *in vivo* studies

showed no evidence of anti-androgenic activity with propiconazole.

Propiconazole was negative in the HTS screens related to thyroid receptor activation (0 of 3 assays). The overall pattern in HTS assays for anti-thyroid activity was generally negative, with some induction of hepatic CYP enzymes and a positive response in one of two thyroid antagonist assays (but at concentrations >50 μM). No *in vitro* assays for direct thyroid receptor effects were available for the EDSP Tier 1 battery, but ample evidence from *in vivo* EDSP Tier 1 assays and published OSRI indicated little to no evidence of thyroid activation nor of anti-thyroid effects. The one study with slight evidence of an anti-thyroid perturbation in rats (Wolf et al. 2006) was negative for morphological endpoints, i.e., there were no effects on thyroid histology or follicular cell proliferation. The liver UDPGT activity increase observed by Wolf et al. (2006) with propiconazole used 1-naphthol (a phenolic compound) as substrate, and 1-naphthol is a primary substrate for UGT1A6 and a minor substrate for UGT1A1. In contrast, the ToxCast assays conducted in human primary hepatocytes, with mRNA levels of human *UGT1A1* as the assay endpoint, were negative for propiconazole. Both UGT1A1 and UGT1A6 have been suggested to play a role in the conjugation of T4 (Vansell & Klaassen 2002), but some inherent differences in the inducibility, substrate specificity and relative contribution of glucuronidation versus sulfation to the catabolism of T4 between rats and humans has been shown (Richardson et al. 2014). Overall, a lack of induction of human UGT1A1 by propiconazole may be considered concordant with its comparatively limited effects on rat thyroid structure and function in the majority of *in vivo* studies. Propiconazole induced mRNA expression of SULT2A1 at one time point in the HTS screens; this sulfotransferase plays a possible role in sulfation and clearance of T3 (Butt & Stapleton 2013). In accordance with the weak evidence for altered thyroid catabolism *in vitro*, no alterations in thyroid histology were observed in 40 CFR Part 158 guideline studies of sub-chronic or chronic propiconazole exposure.

Propiconazole inhibited aromatase *in vitro* in two HTS assays, as well as in the EDSP Tier 1 aromatase inhibition assays that were satisfied by published OSRI. The HTS data demonstrated two out of two assays positive for aromatase inhibition, with AC_{50} values of 2 and 24 μM; published OSRI reported aromatase inhibition at similar concentrations (AC50 values from 1 to 8.25 μM), as well as altered steroidogenesis in two studies at 1–30 μM. The estimated cytotoxicity limit in ToxCast for propiconazole (4.6 μM) indicated that these findings might have occurred in the presence of cytotoxicity. Currently available HTS assays in the ToxCast/Tox21 dataset used in this analysis (USEPA 2014e) did not include any *in vitro* or *in vivo* tests for steroidogenesis, but the results from 40 CFR Part 158 guideline studies and EDSP Tier 1 screens plus OSRI were concordant in that a weak potential to alter steroidogenesis *in vitro* did not translate into a consistent or definitive pattern of effects *in vivo*. Across a wide spectrum of studies, the pattern in mammalian species was negative for effects that would be suggestive of a mechanism related to altered steroidogenesis with propiconazole. The HTS assay data for aromatase inhibition are qualitatively consistent with the EDSP Tier 1 *in vitro* assay data for steroidogenesis. Further, the HTS assay data are consistent with apparent effects *in vivo* on the liver and metabolism. In summary, comparison of the HTS *in vitro* data for aromatase inhibition and AR antagonism with *in vivo* data from the EDSP Tier 1 battery and from guideline toxicology studies highlights that HTS assay data are useful for conservative screening; i.e., although propiconazole inhibits aromatase and demonstrates weak AR antagonism *in vitro*, effects on steroidogenesis or androgen systems were not observed *in vivo*.

Myclobutanil

The HTS assay data and EDSP Tier 1 battery together suggest that myclobutanil may inhibit aromatase to mediate effects on the endocrine system, independent of direct ER and/or AR interactions, but at concentrations or *in vivo* doses that tend to coincide with systemic toxicity (Table 17). The ToxCast AUC ER and AR model scores for myclobutanil were negative, indicating that myclobutanil did not act as a direct agonist or antagonist with the ER or AR. These results were consistent with the EDSP Tier 1 *in vivo* mechanistic assays, as myclobutanil was negative in both the uterotrophic assay for estrogenicity and the Hershberger assay for anti-androgenicity/androgenicity.

The HTS results for myclobutanil indicated aromatase inhibition as a potential MOA for endocrine effects following myclobutanil exposure, with positive responses in both available aromatase inhibition assays (AC_{50} values = 0.672 μM and 5.16 μM), although one of these AC_{50} values exceeded the estimated cytotoxicity limit (4.69 μM). These data suggest an interaction with aromatase, which converts androgens to estrogens, at concentrations that may approach cytotoxicity. If aromatase was inhibited or steroidogenesis was otherwise perturbed *in vivo*, this MOA would not be detected by uterotrophic or Hershberger assays, and the female pubertal assay also may be relatively insensitive to aromatase inhibitors (Marty et al. 1999; USEPA 2007a). Accordingly, these EDSP Tier 1 studies were negative for myclobutanil. Although the results of the two *in vivo* male pubertal studies were inconsistent, i.e., decreased or increased testosterone, depending on the route of administration and different effects on androgen-sensitive organ weights and/or histopathology, the effects that were seen may suggest changes related to altered steroidogenesis. The relatively high-dose levels required to achieve these effects *in vivo*, however, produced concurrent systemic toxicity. Similar results were seen with the mammalian guideline toxicology database, wherein ovarian, testicular and reproductive effects were seen at ≥39, ≥10 and ≥80 mkd, respectively, generally in the presence of BW and/or liver weight changes, as well as histopathological changes.

With respect to thyroid homeostasis, HTS data did not indicate a direct interaction with thyroid hormone receptors, but it did suggest possible induction of hepatic enzymes that could correspond to enhanced thyroid hormone metabolism and clearance (Table 8 and Supplemental File 5). These HTS findings are in agreement with the minor thyroid histopathological changes and markers of hepatic enzyme induction observed in the male pubertal assay in which rats were dosed

via oral gavage. These thyroid histopathological changes were observed in the EDSP Tier 1 studies only when myclobutanil was administered by bolus gavage at a high-dose level (400 mkd); signs of hepatic enzyme induction also were seen at this dose level, e.g., increased liver weight and hypertrophy. The amphibian metamorphosis assay was negative, supporting the conclusion that myclobutanil does not interact directly with thyroid hormone receptors (Supplemental Table 11b). The female pubertal assay was also negative for thyroid effects. Published OSRI did not indicate direct interaction with the hypothalamic–pituitary–thyroid axis (Goetz et al. 2006; Rockett et al. 2006; Tully et al. 2006). The lack of thyroid-related findings is also consistent with the guideline toxicology data, where no effects on thyroid parameters were observed unless the MTD was exceeded, i.e., in a sub-chronic rat study with dietary administration at 3000–10,000 ppm. Thus, the indirect thyroid effects observed *in vivo* in the male pubertal assay resulted only from exposures that exceeded the MTD, with increased T4 metabolism and clearance likely occurring together with systemic toxicity.

Thus, it appears that the HTS assay data, EDSP Tier 1 data, and guideline toxicology data for myclobutanil were concordant, i.e., there was evidence for aromatase inhibition and effects on hepatic metabolism that may correspond to increased enzymatic activity and perturbations of steroid hormone homeostasis. As such, the HTS data appear useful as a bioactivity screen, but toxicokinetics and exposure predictions are necessary to provide enough context to interpret the likelihood of observing hazard *in vivo*.

Bioactivity and exposure: use in prioritization

Science-based prioritization of chemicals for any further endocrine testing under the EDSP should depend on characterization of the margin separating predicted exposure and bioactivity. As an illustration of this concept, the use of high-throughput prioritization methods for the three data-rich triazoles in terms of further characterization of endocrine activity is demonstrated in this manuscript. The key sources of information for this analysis include HTS hazard information from ToxCast, high-throughput human exposure predictions from ExpoCast (Wambaugh et al. 2014) and the publicly available high-throughput toxicokinetic information, i.e., the 95th percentile of the predicted systemic steady-state concentration in humans following oral uptake of a daily 1 mkd administration (USEPA 2014b) (see Methods section and Table 1) that was needed to convert *in vitro* AC_{50} values to human oral equivalent doses for comparison with exposure predictions.

In addition to determining the fit-for-purpose utility of this high-throughput prioritization, a parallel comparison of prioritizations based on high-throughput and more traditional risk assessment approaches is also illustrated. This comparison is critical because it clarifies whether or not HTS-based prioritization would have been conservative and human health protective to enable its use in place of running the EDSP Tier 1 screening battery. Traditional risk assessment approaches included a comparison of projected human exposures based on unrefined models and the oral dose associated with any endocrine-related changes in extensive testing using whole animal models, i.e., in *in vivo* mammalian guideline toxicology studies, as investigated in this work. Acceptance of high-throughput prioritization, particularly for PAIs or chemicals with extensive available data, necessitates that the prioritization system be adaptable to new information sources and similarly protective compared to traditional risk assessment approaches. Figures 2, 3 and 4 present a comparison of high-throughput prioritization versus more traditional risk assessment approaches for endocrine prioritization for myclobutanil, propiconazole and triadimefon. As a tool to facilitate comparisons in these figures, the upper risk assessment panel (RA panel) compares the most recent range of human exposure estimates from EPA risk assessments to a lowest potentially endocrine active dose (LPEAD). The LPEAD is the lowest dose level in *in vivo* mammalian toxicology studies at which there are apical effects potentially related to endocrine systems. Establishing this dose is a conservative exercise for prioritization purposes, and it does not necessarily establish that those effects defining the LPEAD were in fact mediated by a primary endocrine mechanism. Seemingly endocrine-related effects may have resulted from mechanisms secondary to systemic toxicity, e.g., secondary to BW decreases or liver-mediated increases in hormone metabolism and clearance. In such instances, systemic toxicity could precede or occur concomitantly with endocrine-related effects at the LPEAD. Also included in Figures 2–4 is a dotted line indicator of the cytotoxicity caution flag; as indicated previously, this estimated "cytotoxicity limit" is not necessarily a strict "cut-off" for reporting HTS assay data. AC50 values at concentrations that exceed the cytotoxicity caution limit, however, may represent assay positives of little to no biological relevance and may have resulted from assay interference rather than selective *in vitro* activity. At this time, more research is needed to understand how to predict systemic or overt toxicity from *in vitro* data, and the cytotoxicity limit as defined within ToxCast may not necessarily be an indicator of the *in vivo* responses that would occur at equivalent exposure levels.

Triadimefon

Figure 2 RA panel, or values from traditional risk assessment approaches, demonstrates a five orders-of-magnitude separation between the US general population chronic aggregate exposure, a conservative measure of combined dietary and water exposure (USEPA 2009) (see Methods section), and the LPEAD that elicited systemic toxicity and potential endocrine effects in rats in multiple guideline toxicology studies. Specifically, effects observed at the triadimefon LPEAD (1800 ppm or approximately 100 mkd for the rat depending on lifestage) included increased incidence of thyroid follicular adenomas in the chronic rat study (Bomhard & Schilde 1991); decreased male mating behavior in the F1 generation of the multigenerational study (Loser & Lorke 1979); effects on body and liver weights and liver histopathology in multiple studies; increased serum testosterone, increased AGD and decreased male mating behavior in a study similar to a male pubertal assay (Goetz et al. 2007); and decreased serum T4 and other thyroid cellular changes in a non-guideline study (Wolf et al. 2006) (Table 15).

The lower prediction panel of Figure 2 illustrates that greater than five orders of magnitude separate the range of human oral exposures predicted by ExpoCast (Wambaugh et al. 2014) from the distribution of positive responses in ToxCast related to *in vitro* endocrine activity. The distribution of positive assay responses, especially at the lower end of the exposure range, corresponds largely to assay endpoints associated with upregulated hepatic metabolism (see Table 6), which when considered in aggregate, are consistent with high-dose effects of triadimefon that may produce rodent liver histopathological changes and a slightly increased incidence of thyroid tumors following chronic exposure (see Table 12). The assays corresponding to each white bar, or "bin," are listed in Supplemental File 2. The current reference dose used in EPA risk assessments for triadimefon, 0.034 mg/kg-bw/d, is based on the no-observed-adverse-effect level (NOAEL) in the oral sub-chronic neurotoxicity study (3.4 mkd) with hyperactivity observed at higher doses; hyperactivity has also been observed in rats 30 min to 2 h following exposure to 50–60 mkd triadimefon (Crofton 1996). Thus, the risk assessment that was focused on the traditional endpoints and lowest dose effects is protective for any potential effects on the endocrine system, as these effects occur at much higher doses, if at all.

The white bars indicate the frequency or number of positive assay AC_{50} values at oral equivalent dose ranges or "bins". Detailed review of the bins occurring at oral equivalent doses lower than the cytotoxicity caution flag (1.68 μM or 5.13 mkd) corresponded to hepatic catabolism assays, namely NVS_ADME_rCYP2B1 (1.54 mkd) and NVS_ADME_hCYP2C19 (3.62 mkd) (see Table 6 and Supplemental File 2). Indeed, 28 of the 36 endocrine-related ToxCast AC_{50} values indicate effects on liver metabolism, generally at concentrations that exceed the cytotoxicity limit for triadimefon, which may indicate potential implications for systemic thyroid hormone clearance (refer to Table 6 for endocrine-related positive HTS assay endpoints).

The estimated margin between the 95% confidence limit on the ExpoCast exposure prediction distribution and the lower end of the distribution of endocrine-related assay results from ToxCast is very similar to the safety margin between the EPA aggregate chronic exposure estimates and an effect level (LPEAD) from *in vivo* studies of endocrine relevance. The positive assay endpoints of endocrine relevance for triadimefon are largely related to hepatic catabolism with potential significance for increased systemic thyroid hormone clearance. This is concordant with the rat thyroid histological changes observed *in vivo* (Bomhard & Schilde 1991; Rockett et al. 2006), plus decreased serum T3 and T4 in the rat (Rockett et al. 2006; Wolf et al. 2006; Goetz et al. 2007) and increased hepatic glucuronyltransferase activity in the rat (Wolf et al. 2006) with 1800 ppm dietary exposure (100 mkd in rats). These *in vivo* data suggest that high-dose, prolonged exposures in rats result in liver toxicity with concomitant potential for increased thyroid hormone clearance that may correspond to thyroid histological changes *in vivo*, but at doses that may also result in systemic toxicity and that exceed exposure thresholds for neurotoxicity with triadimefon. Thus, using neurotoxicity for the point of departure protects for all other effects, including the potential perturbation of thyroid and steroid synthesis pathways.

Triadimefon appears to be of low priority for further endocrine activity screening, based on the large margins of separation between HTS predictions of exposure and effects (five orders of magnitude) and similar margins in a more traditional approach to estimating possible exposure and hazard (five orders of magnitude).

Propiconazole

For propiconazole, a closer examination of the HTS assays that are binned together in the graph (Figure 3) at the lower dose levels (0.03–0.514 mkd) indicates that the positive results for propiconazole at lower estimated doses (Bins 1–4) were all assay endpoints related to liver metabolism or transporter enzymes (Table 7 and Supplemental File 2). Because no evidence of thyroid effects was observed in the *in vivo* 40 CFR Part 158 guideline studies with propiconazole (Table 3), these *in vitro* activities related to thyroid hormone catabolism are considered adaptive changes in the liver that have no effect on the endocrine-relevant risk evaluation for propiconazole. At slightly higher concentrations (Bins 5–8 in Supplemental File 2; equivalent to 1.486–4.999 mkd), the majority of the assays are again related to thyroid hormone catabolism, with the exception of one assay related to estrogenic effects and one indicating CYP19A1 (aromatase) inhibition related to steroidogenesis. As discussed earlier, the other HTS assay for aromatase inhibition was positive at 23.56 μM (21.4 mkd), which is well above the estimated cytotoxicity limit. All the other bins (5.3–64.3 mkd) are above the estimated cytotoxicity limit (4.6 μM = 4.15 mkd) for propiconazole and are thus of lower relevance, considering the potential for confounding as a result of cytotoxicity in the HTS assays (dotted line in Figure 3).

Approximately four orders of magnitude separate the range of predicted human oral exposures in ExpoCast (Wambaugh et al. 2014) from the lowest estimated effect level (Bin 1; 0.03 mkd) of any ToxCast result for propiconazole. Greater than five orders of magnitude separate the ExpoCast estimated exposures from the lowest endocrine-relevant response from ToxCast (Bin 5; 1.486 mkd).

The LPEAD, represented by a triangle in the top panel of Figure 3, represents the dose from the two-generation reproduction study in rats with propiconazole (2500 ppm =200 mkd) that is pertinent to endocrine activity evaluation. Effects at this dose included parental and offspring effects such as decreased BW and offspring survival; there were no effects on reproduction (USEPA 2014d). The separation between the LPEAD and the US general population chronic aggregate exposure (dietary and water combined; 0.00743 mkd, see Methods section) (USEPA 2014d) represents a margin of greater than four orders of magnitude.

In summary, the estimated margin between ExpoCast human exposure estimates and endocrine-related HTS assay results is very similar to the margin separating EPA aggregate chronic exposure estimates and an effect level (LPEAD) from *in vivo* mammalian studies of possible endocrine relevance. Further, the types of effects seen at lower estimated doses in

Table 18. AUC model and IBER values for the three triazoles.

	AUC Model Scores* and IBER† values					
Chemical	ER agonist	ER antagonist	ER IBER	AR agonist	AR antagonist	AR IBER
Myclobutanil	0	0	1,000,000	0	0	1,000,000
Propiconazole	0	0	1,000,000	0	0.111	643,056
Triadimefon	0.0095 (negative; below threshold)	0	1,000,000	0	0	1,000,000

*AUC model, area-under-the-curve model for ER or AR pathway activity.
†IBER, integrated bioactivity and exposure ranking. From Browne, et al. (2015) and USEPA (2014b).

the ToxCast assays (hepatic enzyme induction) are of low relevance to endocrine pathways given the lack of thyroid effects in the *in vivo* profile of propiconazole. These *in vitro* HTS results may point to the potential for adaptive hepatic effects, which were seen at lower doses, e.g., 50 mkd, in *in vivo* rodent studies with propiconazole. Propiconazole appears to be of low priority for further endocrine activity screening based on the large margins of separation between HTS predictions of exposure and effects (five orders of magnitude) and similar margins in a more traditional approach to estimating possible exposure and hazard (four orders of magnitude).

Myclobutanil

The main panel of Figure 4 shows the range of predicted human oral exposures for myclobutanil from ExpoCast [median, upper 95% confidence limit from Wambaugh et al. 2014] versus the frequency distribution of possible endocrine-related bioactivity hits in ToxCast, represented as oral equivalent doses (mkd). Seven positive ToxCast assay responses occur in Bins 1–4 at oral doses equivalent to ~1–10 mkd, which are below the cytotoxicity caution flag (4.69 μM), represented as a dotted line. These positive responses correspond to assay endpoints related to aromatase inhibition (Bin 1; $AC_{50} = 1.18$ mkd) and hepatic enzyme induction (Bins 1–4), consistent with induction of hepatic metabolism observed with myclobutanil *in vivo*.

Figure 4 RA panel shows the separation between the chronic aggregate exposure estimate from the US general population (0.005013 mkd) (see Methods section) (USEPA 2007b, 2008) and the LPEAD (9.84–12.86 mkd). The LPEAD, represented by a triangle in the top panel of Figure 4, represents the lowest dose of myclobutanil from the chronic rat study that is relevant for endocrine evaluation, based on male reproductive endpoints *via* aromatase inhibition and/or liver toxicity resulting in altered liver hormone homeostasis in rats.

The estimated margins betweenthe HTS prioritization approach and the traditional risk assessment approach were similar. There were five orders of magnitude between the oral doses corresponding to the ExpoCast 95% confidence limit on predicted human exposure and the lowest oral equivalent doses for endocrine-related HTS bioactivity. In comparison, the separation between the LPEAD and the US general population chronic aggregate exposure was greater than three orders of magnitude.

Both these margins demonstrate conservative separation between predicted exposure and potential bioactivity. Overall, myclobutanil appears to be of low priority for further endocrine activity screening, based on these large margins between HTS-based predictions of exposure and effects, as well as a more traditional approach to estimating exposure and hazard.

Comparison to the integrated bioactivity and exposure ranking approach

An integrated bioactivity and exposure ranking (IBER) approach was developed by EPA (USEPA 2014b; Browne et al. 2015) as a means of prioritizing the universe of approximately 10,000 chemicals that could be potentially subject to EDSP evaluation. Bioactivity predictions for the IBER approach to prioritization are derived from pathway-based models for ER and AR that utilize multiple receptor-based assay endpoints using assay data from ToxCast and Tox21. AUC model scores and IBER scores for the three triazoles are shown in Table 18.

The current ER-based bioactivity model, or the ToxCast ER AUC model, incorporates information from 18 ER pathway-based assays (16 agonist and/or receptor binding assays, and two antagonist assays) and employs thresholds for positive response scores, with the ER agonist and antagonist AUC model score threshold $\geq$0.1 for a positive. The AR-based bioactivity model, or the ToxCast AR AUC model, similarly incorporates information from nine AR pathway assays (seven agonist and/or receptor binding assays, plus two antagonist assays) and employs thresholds for positive response scores, with the AR agonist threshold score $\geq$0.1 for a positive, and the AR antagonist threshold score $\geq$0.05 for a positive. All three of these triazoles were negative for ER agonist, ER antagonist, and AR agonist pathway activity using the AUC model approach. Propiconazole was a weak positive for AR antagonist activity using the AUC model approach.

The IBER score is the ratio between minimum positive activity, i.e., the oral equivalent exposure for threshold activity in the pathway model, and the maximum (95th percentile) predicted human exposure using high-throughput methods, i.e., ExpoCast (Wambaugh et al. 2014). To derive this score, the chemical concentration predicted to result in the minimum (threshold) model activity is converted to an oral equivalent using high-throughput toxicokinetic modeling (USEPA 2014b). This value is then divided by the 95th percentile of maximum predicted human exposure. This ratio is the IBER score. A score of 10^6 is a default for a negative score, arising from a lack of AUC model activity, i.e., cannot divide zero by a predicted exposure.

These three triazoles demonstrated negative ER IBER scores because they did not have any associated ER pathway activity in the model. Similarly, myclobutanil and triadimefon demonstrated negative AR IBER scores. Propiconazole demonstrated a weak positive AR antagonist AUC score (0.111), but the predicted 95th percentile exposure (1.77 E-6 mkd; Table 2) is so

low that the AR IBER value reflects a wide margin of separation (643056), and thus a lack of priority. The IBER score for propiconazole is the sixth IBER score from the bottom of the list for positive AR IBER scores (USEPA 2014b). Strong positive IBER scores for AR were demonstrated for testosterone propionate (0.000101) and selective AR modulators, such as 17β-estradiol (110) and linuron (4357), with scores reflecting a combination of potency and potential for human exposure. Thus, the IBER system suggests that these three triazoles would be of little to no priority for further ER or AR pathway-related screening or testing under the EDSP.

Discussion

This case study examined the concordance among three lines of evidence for endocrine activity for a small set of well-studied triazole fungicides: (1) HTS results, (2) EDSP Tier 1 assay results (plus published OSRI), and (3) 40 CFR Part 158 guideline mammalian toxicology studies. In addition, human exposure estimates from a high-throughput modeling approach (ExpoCast) were compared with oral equivalent doses for positive findings in ToxCast/Tox21 HTS assays to assess the relative priority of these compounds for further endocrine screening and testing. After compiling this information for triadimefon, propiconazole and myclobutanil, the four critical questions listed below are addressed:

1. Are HTS assay data currently sufficient for an endocrine prioritization task, and does the current HTS battery (ToxCast/Tox21) provide appropriate biological coverage for each of the sub-types of endocrine pathways (estrogen, androgen, thyroid and steroidogenesis)?
2. Are HTS information, guideline toxicology data, and the EDSP Tier 1 assays sufficiently concordant in this example such that HTS prioritization would be possible? In other words, are there major biological gaps or false negatives in HTS that would have affected EDSP evaluation and risk assessment for these three PAIs?
3. Is prioritization based on high-throughput exposure and bioactivity predictions as protective of human health as traditional risk assessment approaches?
4. In the absence of the EDSP Tier 1 battery, could human health-protective prioritization decisions be made regarding the need for any additional hazard and exposure information using HTS prioritization alone or in combination with the available 40 CFR Part 158 guideline toxicology study information?

Regarding biological coverage in HTS assays for each of the sub-types of endocrine pathways (estrogen, androgen, thyroid and steroidogenesis)

The HTS assays currently available via ToxCast and Tox21 provide bioactivity pathway coverage for ER- and AR-mediated activity, effects on steroidogenesis via inhibition of aromatase and potential effects on thyroid hormone homeostasis via thyroid hormone receptor assays and markers of nuclear receptor-mediated hepatic catabolism. The question of suitable ToxCast assay coverage to predict *in vivo* outcomes in the EDSP Tier 1 battery was previously investigated using ToxCast Phase I assay information and led to the conclusion that, at that time, enough assay data for predictive modeling purposes was likely only available for ER and AR pathways (Rotroff et al. 2013). In agreement with this earlier assessment, the current case studies with three data-rich triazole fungicides indicated that sufficient HTS assays are available to cover estrogen, anti-estrogen, androgen and anti-androgen effects, based on their ability to predict the potential (or lack of potential) for estrogen and androgen pathway effects in the 40 CFR Part 158 guideline studies and EDSP Tier 1 testing of these molecules.

There are some known gaps in the current ToxCast/Tox21 assay battery related to steroidogenesis and thyroid pathways (Rotroff et al. 2013; Paul et al. 2014) that limit current efforts to develop a predictive system for these endocrine activities, but predictive models for these pathways are the subject of ongoing research (USEPA 2014b). Despite known limitations inherent to current screening assay technologies in the HTS set, combining HTS information with 40 CFR Part 158 guideline toxicity testing and published OSRI comprises a viable strategy for determination of potential endocrine activity and the presence of possible data gaps that could be addressed by any further endocrine activity evaluation for these PAIs. Importantly, for data-rich chemicals, thyroid and steroidogenesis information can be inferred from guideline and published non-guideline toxicology studies as a second step in the prioritization process. In line with reduction and refinement of animal resources, this type of approach would also focus further screening and testing on clear areas of unknown hazard, and this reflects the approach taken by EPA in recently released weight-of-evidence documents for evaluating the Tier 1 data for the EDSP List 1 chemicals (USEPA 2015a).

At the time this manuscript was written, the HTS battery of ToxCast and Tox21 included two assays to predict aromatase inhibition as indicators for potential impact on steroidogenesis, indicating a potential screening gap for other enzymatic or transport functions necessary for timely steroid production (Stoker et al. 2000; Rotroff et al. 2013). HTS using the H295R steroidogenesis model has been conducted, and the data were publicly released in November 2015; however, these results are not part of the current manuscript. Further, a systems biology model for steroidogenesis similar to the relatively new models for integrating multiple assay predictors for the ER and AR pathways is not yet available, but this is anticipated as part of ongoing work by EPA and the Tox21 initiative (USEPA 2014b). At this time, it is clear that in the case of these PAIs with large toxicology databases, a weight-of-evidence approach that combines HTS information on aromatase inhibition and *in vivo* data from guideline toxicology studies would have provided sufficient data for prioritization for EDSP. Once a more complete systems biology model is available for steroidogenesis that covers a wider range of steroidogenic enzymes, and the predictive accuracy of this model can be compared to results from aromatase and steroidogenesis assays, in addition to *in vivo* pubertal and multi-generation study results, a further assessment of the sufficiency of HTS

methods for predicting perturbed steroidogenesis, without considering other data sources, will become possible.

Another key limitation of current HTS is the absence of screening assays for all the known molecular-initiating events of thyroid perturbation. Thyroid hormone homeostasis is regulated at multiple nodes, including synthesis in the thyroid gland as modulated by the sodium-iodide symporter and thyroperoxidase; release and transport of thyroid hormones systemically; peripheral conversion of T4 to T3 by deiodinases; increased systemic clearance related to upregulation of nuclear receptor-regulated pathways of thyroid hormone metabolism and transport; systemic transport via binding to serum proteins; and, potentially, interactions with thyroid hormone nuclear receptors in target cells (Murk et al. 2013). As of this analysis, ToxCast/Tox21 coverage for direct thyroid receptor interaction is available, and some data are available for nuclear receptor-mediated and thyroid hormone-related hepatic catabolism. In November 2015, additional assays that detect possible *in vitro* inhibitors of thyroperoxidase were added to the ToxCast assay suite (Paul Friedman et al. 2016). HTS data are not available in the ToxCast/Tox21 assay results for many other bioregulatory nodes related to thyroid function (Paul et al. 2014), although the relative importance of these additional nodes and the proportion of thyroid-perturbing agents that act via these nodes is not fully known at this time. Additional HTS assays for steroidogenesis and thyroid perturbation would support development of systems biology models to predict endocrine system interactions via many different MOAs, outside of aromatase inhibition and effects on hepatic catabolism of thyroid hormones.

Similar to our conclusion for steroidogenesis pathway coverage, a weight-of-evidence approach that combines HTS information on hepatic catabolism endpoints related to thyroid hormone homeostasis and *in vivo* data from guideline toxicology studies would have provided sufficient data for prioritization for EDSP for these three triazoles. Once a more complete systems biology model is available for thyroid hormone regulation, including thyroidal and extrathyroidal endpoints (Murk et al. 2013), and the predictive accuracy of this model can be compared with results from *in vivo* study results, further assessment of the sufficiency of HTS methods for predicting perturbed thyroid hormone homeostasis, without inclusion of other data sources, will move forward.

Regarding concordance of HTS information, guideline toxicology data and the EDSP Tier 1 battery plus published OSRI for each triazole

A key result of this effort is the finding that HTS provides an informative line of evidence for endocrine prioritization that is concordant with the EDSP Tier 1 assays plus published OSRI and the 40 CFR Part 158 guideline toxicology study set for these three triazole fungicides. The combined ToxCast and Tox21 HTS assay battery provides multiple, orthogonal endocrine bioactivity predictions related to the ER and AR pathways; this multi-assay coverage has enabled the development of systems biology models to integrate these data for increased reliability based on greater accounting for sources of assay interference and degree of concordance across technologies (Rotroff et al. 2014; USEPA 2014b; Browne et al. 2015). The resultant models, referred to as the ToxCast ER and AR AUC models, enable greater utilization of these receptor pathway data because false positives in various assay technologies are less likely to affect the overall model result. The ToxCast ER AUC model has been suggested as a replacement for screens in the EDSP Tier 1 battery, including the ER binding and transactivation assays and the uterotrophic assay, based on the high predictivity of this model for uterotrophic assay study results (USEPA 2014b, 2015b). The results of the ER and AR AUC models for estrogenic, anti-estrogenic, androgenic and anti-androgenic effects were concordant with the outcomes of the EDSP Tier 1 assays for the three triazole fungicides evaluated in this manuscript. All three triazoles are considered negative for ER pathway activity in HTS.

Triadimefon and myclobutanil were negative for any effects on AR binding in the assays and in the AR AUC models. Propiconazole demonstrated a very high Ki binding constant for AR (indicating a low affinity) and also demonstrated a marginally positive anti-androgenic ToxCast AR AUC model score (0.111) based on AR antagonism and binding, but at concentrations that are likely irrelevant to *in vivo* models. This anti-AR score was concordant with OSRI study results, in which four published *in vitro* studies (Table 10) provided evidence for antagonism of the AR but only at relatively high concentrations (3–96 μM). This weak *in vitro* signal, however, did not translate into any evidence of anti-androgenic effects with propiconazole based on *in vivo* studies. Propiconazole was negative in EDSP Tier 1 assays and/or OSRI that satisfied requirements for the Hershberger assay (Kjaerstad et al. 2007; Taxvig et al. 2008) and the male pubertal assay (Tully et al. 2006; Goetz et al. 2007), and no evidence of adverse anti-androgenic effects exists in the 40 CFR Part 158 guideline toxicology database.

In summary, the HTS results and EDSP Tier 1 battery did not suggest primary effects on ER or AR pathway signaling for any of the three triazoles. Despite a small number of weak positive results in HTS anti-estrogen assays and in the anti-androgen assays, all three triazoles were negative in the Hershberger assay, and they failed to demonstrate consistent effects on estrogenic signaling in either a female pubertal assay design (Rockett et al. 2006) or in the uterotrophic assay (though the uterotrophic study was waived for triadimefon). These comparisons indicate that the ER and AR AUC models would have been reasonable substitutes for the ER- and AR-related assays in the EDSP Tier 1 screening battery for these triazoles.

All three triazoles inhibited aromatase (CYP19A1) in both assays available in the HTS battery. This is consistent with prior knowledge that triazoles have the potential to inhibit certain isoforms of CYP enzymes, based on the common fungicidal MOA for triazoles via inhibition of Cyp51 in fungi (Trosken et al. 2006b). These HTS results correctly correspond to the *in vitro* aromatase assay results in the EDSP Tier 1 battery: all three triazoles inhibited aromatase in the OCSPP 890.1200 guideline test and/or in various publications deemed OSRI. Though the HTS predictions and EDSP Tier 1 aromatase and steroidogenesis assays were in agreement, the apical outcomes observed in the *in vivo* portion of the EDSP Tier 1

Table 19. AC_{50} values in HTS aromatase inhibition assays for the three triazoles

	Triadimefon		Propiconazole		Myclobutanil	
Assay endpoint name	μM	OED*	μM	OED	μM	OED
NVS_ADME_hCYP19A1	2.06	0.67	2.24	2.5	0.67	0.38
Tox21_Aromatase_Inhibition	36.8	12	23.8	26	5.16	2.9

*OED, oral equivalent dose, mkd, calculating using the Css95% (Table 1).

battery varied by triazole. Thus, *in vitro* inhibition of aromatase may provide important screening information, but it does not necessarily translate into endocrine-mediated effects in more complex study designs, such as the male and female pubertal assays or two-generation reproduction studies. As shown in Table 19, the AC_{50} values in both HTS aromatase inhibition assays were lower for myclobutanil than for triadimefon or propiconazole. The EDSP Tier 1 plus published OSRI results for aromatase inhibition were concordant with this pattern: myclobutanil had an $IC_{50} = 0.1$ μM in a guideline aromatase assay, compared with values of 1–32 μM for propiconazole and triadimefon (Tables 9–11). All three triazoles inhibited steroidogenesis in H295R cells (Goetz et al. 2009), but further delineation of relative potency was not possible from this published assay. The direction of change in testosterone or estradiol levels varied by compound at lower concentrations (1–3 μM), but these triazoles tended to decrease concentrations of both estradiol (propiconazole and myclobutanil) and testosterone (all three triazoles) at concentrations >3 μM (Goetz et al. 2009).

For triadimefon, although it inhibited aromatase *in vitro*, the direction of effects on testosterone production was not consistent between *in vitro* and *in vivo* assays, possibly because any *in vivo* effects were obscured by systemic toxicity. As discussed previously in Triadimefon section, both increases and decreases in testosterone were reported *in vivo*, and concurrent effects were seen in the presence of systemic toxicity, including decreased BW and litter survival (Rockett et al. 2006; Goetz et al. 2007). Effects of triadimefon on reproductive success for F1 male rats at 1800 ppm in a study similar to a male pubertal assay (Goetz et al. 2007) and in two-generation reproduction studies (Loser & Lorke 1979; Eiben 1984) resulted in decreased male reproductive behavior and may have reflected a non-endocrine inhibition of dopamine reuptake with triadimefon (Crofton 1996). This assessment is consistent with the weak potential for triadimefon to inhibit steroidogenesis and the lack of any effects on testis or ovary micropathology or sperm parameters. The simultaneous demonstration of systemic toxicity *in vivo* at doses that may have caused neurotoxicity underscores the idea that aromatase inhibition may not have occurred in rat studies *in vivo,* or may have occurred, but only at doses above those that produced other non-endocrine toxicities.

Propiconazole inhibited aromatase *in vitro*, but it failed to elicit anti-estrogenic or androgenic responses that would be consistent with aromatase inhibition *in vivo* in rodent models of the EDSP Tier 1 assays or in the 40 CFR Part 158 guideline toxicology studies. Propiconazole did not produce any consistent, treatment-related changes in serum testosterone or estradiol levels in any mammalian species in the *in vivo* EDSP Tier 1 assays (Table 10). Propiconazole, as described in this case study, had no effects on reproduction in rats at dose levels up to 2500 ppm (200 mkd). A slightly lower number of F2A pups delivered, pups delivered viable, and pups surviving to postnatal day (PND) 4 did not match precisely the effects in F2B litters, in which numbers of pups delivered were similar to controls, but survival was slightly lower at PND7–21 (Borders & Salamon 1985). This minor effect in the F2 litters with propiconazole is considered a marker of offspring systemic toxicity, as indicated by large BW deficits at this high dose in dams and in pups.

Myclobutanil inhibited aromatase *in vitro*, and some of the effects observed *in vivo* might have been a result of aromatase inhibition. These effects, however, were observed only at doses that coincided with liver or systemic toxicity. Dietary myclobutanil exposure (2000 ppm or approximately 153.5 mkd) resulted in androgenic effects – including increased AGD in male pups, increased testes weight, increased testosterone levels and decreased male reproductive success (without any effect on sperm parameters or histology of androgen-sensitive organs) – in a published male pubertal study cited as OSRI (Goetz et al. 2007). These effects were contrary to the anti-androgenic effects observed in the guideline male pubertal assay that employed gavage exposures (Marty et al. 2011). At a high-dose level of 400 mkd in the guideline male pubertal assay (Marty et al. 2011), anti-androgenic effects including decreased testosterone, slightly delayed PPS and decreased androgen-sensitive organ weights (in the absence of histopathology changes) were observed (Table 11). It is noteworthy that the dose levels at which these effects were observed also accompanied by hepatic changes, including increased liver weights and hypertrophy in both the studies.

Myclobutanil increased AGD in female pups, delayed VO and increased ovary weights (with no effects on serum estradiol or estrous cyclicity) in a published female pubertal assay cited as OSRI (Rockett et al. 2006). In contrast, a guideline *in vivo* EDSP Tier 1 pubertal assay in female rats (Marty et al. 2011) with myclobutanil demonstrated a lack of effects on estradiol levels, VO or weight and histology of estrogen-sensitive organs (Table 11). The 40 CFR Part 158 guideline chronic rat studies with myclobutanil at dose levels ≥200 ppm demonstrated increased incidences of testicular atrophy, decreased testes weight, aspermatogenesis and hypospermia in the epididymides (Table 14). In a rat reproduction study, myclobutanil exposures of 1000 ppm increased testis and prostrate atrophy, decreased spermatozoa in the testis and epididymides (P2 generation only), decreased reproductive parameters and increased numbers of stillborn pups following mating. Other sensitive endpoints typically altered by aromatase inhibitors were not affected, e.g., estrous cyclicity and ovarian histopathology (refer to Myclobutanil section), and moreover, the contribution of liver effects to altered steroid

Table 20. AC_{50} values in HTS assays for UGT1A1 and SULT2A1 induction.

	Triadimefon		Propiconazole		Myclobutanil	
Assay endpoint name	μM	OED*	μM	OED	μM	OED
CLZD_SULT2A1_48	8.29	2.7	7.44	8.2	NA	NA
CLZD_UGT1A1_24	9.66	3.2	NA†	NA	14.6	8.3

*OED, oral equivalent dose, mkd, calculating using the Css95% (Table 1).
†NA, tested but negative in this assay.

metabolism cannot be excluded, as liver changes were seen at similar or lower dose levels. Thus, these results, while concordant with *in vitro* data, may be confounded by other MOAs, e.g., liver metabolism, contributing to the *in vivo* effects observed.

Regarding thyroid effects, all three triazoles were negative for direct effects on the thyroid hormone receptor in the HTS results. Concordant with the HTS results, the amphibian metamorphosis assay was negative for thyroid-mediated effects with propiconazole and myclobutanil. The AMA assay was waived for triadimefon, but the wider database for triadimefon suggested a weak potential for effects on rodent thyroid at 1800 ppm in the chronic rat study, a dose that also increased liver weights and decreased BW (Bomhard & Schilde 1991). Further data with triadimefon indicated that the rodent thyroid effects most likely were secondary to increased clearance of thyroid hormones, via induction of liver metabolism enzymes. In addition, the effects on the thyroid via this indirect mechanism were more consistently observed in the database for triadimefon than for propiconazole or myclobutanil.

All three triazoles likely affected nuclear receptor activation (discussed further below) based on increased expression of some specific Phase I enzymes, e.g., CYP2B, CYP3A and Phase II conjugation enzymes (e.g., UGT1A1 and/or SULT2A1) that could modulate thyroid hormone catabolism (Rotroff et al. 2010; Paul et al. 2013). Differences between the three triazoles, however, were identified in the potential for thyroid changes from *in vivo* rat studies that were part of the 40 CFR Part 158 guideline studies and the *in vivo* studies that were part of the EDSP Tier 1 plus published OSRI data. Triadimefon produced histologic changes in the rat thyroid in a chronic rat study, including cystic hyperplasia and a very slight increase in follicular cell adenomas at 1800 ppm (Bomhard & Schilde 1991). Effects with triadimefon in a sub-chronic rat study were limited to increased thyroid weight in females only at 2000 ppm (Mohr 1976), and a published study similar to a female pubertal assay demonstrated mild follicular hypertrophy in females at 1800 ppm (Rockett et al. 2006), but no effects were seen in males at 1800 ppm in a published study similar to the male pubertal assay (Goetz et al. 2007). A 90-d mechanistic study demonstrated thyroid histopathological changes, including follicular cell hypertrophy, colloid depletion and cell proliferation at 30 d, along with decreased T4 and T3 at 4 and 30 d, but not at 90 d (Wolf et al. 2006). These authors suggested that the mechanism for the observed thyroid effects was via increased T4 clearance resulting from induction of UGT1A1 and similar isoforms, but this induced catabolism was likely not of sufficient magnitude to result in a measurable increase in TSH levels at the time points that were examined. Myclobutanil and propiconazole exposures were not associated with any changes in thyroid weight or histology in rat sub-chronic and chronic studies; they were also not associated with any increased incidence in thyroid tumors. Myclobutanil exposure increased relative thyroid weights and increased the number of small follicles in the sub-chronic rat study at 3000–10,000 ppm, doses that exceed the MTD for myclobutanil. The study by Wolf et al. (2006) demonstrated decreased T4 and/or T3 at 4 and 30 d of propiconazole or myclobutanil exposure at the same doses that increased liver weights, but with no increases in TSH and no histologic changes in the thyroid at any measured time-point (4, 30 or 90 d). Thus, all three triazoles may upregulate hepatic catabolism of T4 and T3 in the rat, but with varying potential to affect downstream key events related to the thyroid gland at the *in vivo* doses tested.

The HTS assay results for enzyme activities that are known to be related to thyroid hormone conjugation and clearance are summarized in Table 20 for the three triazoles. UGT1A1 is one enzyme responsible for conjugation and subsequent biliary excretion of T4, and SULT2A1 is one enzyme responsible for conjugation and clearance of T3 (Butt & Stapleton 2013). Triadimefon induced both UGT1A1 and SULT2A1, whereas propiconazole (SULT2A1) and myclobutanil (UGT1A1) each induced only one of these enzymes. The AC_{50} values for each of these activities were similar across the three triazoles. From this limited HTS dataset, it is uncertain whether greater induction of these conjugating enzymes for T4 and T3 by triadimefon may correlate with its slightly greater tendency to ultimately produce histological changes in the thyroid of rats compared to propiconazole and myclobutanil. Future development of systems biology models of thyroid hormone metabolism may increase understanding of how the potency and efficacy of chemical effects in *in vitro* model systems may relate to thyroid homeostasis *in vivo*; currently such a model is unavailable.

The *in vitro* HTS bioactivity predictions suggest that myclobutanil, propiconazole and triadimefon target the liver, likely via interaction with nuclear receptors (CAR, PXR and/or possibly others) that act as transcriptional activators of Phase I and II metabolism enzymes, as well as hepatic transport proteins. Although the majority of the nuclear receptor reporter assays and CYP or Phase II enzyme induction assays in ToxCast are for human forms, the 40 CFR Part 158 guideline toxicity studies and EDSP Tier 1 testing protocols are primarily based on rat or mouse results. Depending on the assay endpoint, some species differences can occur in the activation profile for a particular chemical structure. For example, published studies have demonstrated that propiconazole and a closely related triazole (etaconazole) are potent activators of mouse and rat CAR in a CAR3 reporter assay, but only weak activators of human CAR (Omiecinski et al. 2011; Currie et al.

2014). In accordance with the HTS results, it is possible that specific nuclear receptors, e.g., CAR and/or PXR, may also be operative in the liver responses to each of these three triazole fungicides, and this response may be divergent by species (Omiecinski et al. 2011; Paul et al. 2013). However, the pattern of induction of Phase I and Phase II metabolism enzymes and liver transporters for the three triazoles, based primarily on human isoforms tested in ToxCast Phase I, is concordant with the established sub-chronic and chronic studies in rodents, where the liver is a key target organ. The EDSP Tier 1 assays produced concordant results, including increased microsomal enzyme induction, liver weights and incidence of centrilobular hypertrophy with the three triazoles. While these effects in the liver may not be directly related to the endocrine systems being evaluated in EDSP Tier 1 testing, they are important in terms of (1) a possible role of liver enzyme activities in steroidogenesis (either via induction or inhibition); (2) a possible role of liver enzyme induction in catabolism of thyroid hormones; and (3) the possible role that liver toxicity may play in contributing to systemic toxicity and/or establishing the MTD for these three triazoles.

In conclusion, the HTS data were useful as a conservative screen to delineate the endocrine pathways that were not affected by these triazoles, i.e., the ER, AR and TR pathways, as well as steroidogenesis and thyroid hormone metabolism pathways that may be affected. *In vitro* positives related to steroidogenesis and thyroid hormone metabolism did not always correspond to effects *in vivo* in higher-tier studies, but these "false positives" can be tolerated in screening because further screening and testing, along with the potential for exposure, can be used to put these bioactivity predictions in the appropriate context.

Regarding prioritization for endocrine testing based on high-throughput human exposure and bioactivity predictions

In addition to examination of the agreement between these three separate lines of evidence for endocrine activity screening, we present in this manuscript a visualization tool (Figures 2–4) to facilitate endocrine prioritization decisions based on high-throughput human exposure and bioactivity predictions. The goal of this visualization is to simultaneously compare high-throughput exposure and bioactivity predictions and estimates of exposure and hazard using more traditional risk assessment approaches, and then to determine whether the margin of separation would have been similar using either of the two approaches and/or conservative enough to be used in prioritization. EPA has suggested using such an approach, i.e., integrated bioactivity and exposure ranking (IBER), when limited data are available to characterize the potential for a chemical to demonstrate endocrine activity (USEPA 2014b). Thus, Figures 2–4 provide key examples of how such an approach might have worked had it been available to the EDSP during the evaluation of List 1 chemicals. The case studies presented herein suggest that prioritization using high-throughput predictions would have been human health protective for these PAIs.

In the upper panel of Figures 2–4, the distance separating the LPEAD (based on mammalian *in vivo* study outcomes) and EPA chronic aggregate exposure estimates is illustrated. This degree of separation is compared to the outputs available from high-throughput endocrine screening (ToxCast/Tox21) and high-throughput human exposure estimates (ExpoCast) for that molecule. For triadimefon, the separation of the high-throughput values (greater than five orders of magnitude) was similar to the separation of EPA exposure estimates and the LPEAD values (greater than four orders of magnitude). For propiconazole, the separation of the high-throughput values was virtually the same as EPA exposure estimates and the LPEAD value (each greater than four orders of magnitude). For myclobutanil, the separation via the high-throughput values (greater than five orders of magnitude) was slightly greater than the separation between EPA exposure estimates and the LPEAD (greater than three orders of magnitude), but both demonstrated a high degree of conservatism for prioritization and risk assessment, respectively.

Consideration of these safety margins for potential endocrine activity of the three example triazoles that were evaluated indicates two important conclusions. First, the margins of separation attained using HTS bioactivity and human exposure methods provide similar margins to those using traditional *in vivo* toxicity studies and EPA human exposure estimates. Second, based on the large margins of separation between predicted bioactivity and exposure, triadimefon, propiconazole and myclobutanil should be of low priority for additional endocrine testing.

In reviewing the margins separating the LPEAD and the chronic aggregate exposure estimate, it should be underscored that traditional EPA human health risk assessment processes would have been protective of any endocrine activity observed in *in vivo* mammalian studies in the EDSP Tier 1 battery or on endocrine-related endpoints in 40 CFR Part 158 guideline toxicology studies. Triadimefon provides a clear example of a chemical for which any potentially endocrine-relevant effects clearly do not affect the human health risk assessment because other effects occur at much lower doses. The human health risk assessment for triadimefon is based on increased motor activity in rats that received a sub-chronic exposure, with a *lowest-observed-adverse-effect level* (LOAEL) of 54.6 mkd and a NOAEL of 3.4 mkd (USEPA 2009). The NOAEL for hyperactivity is approximately 3% of the LPEAD presented in Figure 2. Triadimefon is thought to block reuptake of dopamine, leading to hyperactivity in rodents (Crofton 1996). A chronic reference dose is derived by dividing this NOAEL by 10 for rat to human interspecies extrapolation and another factor of 10 for intraspecies variability. The Food Quality Protection Act safety factor has been set to "1" because no increased prenatal or postnatal susceptibility was identified in a developmental neurotoxicity study with rats. Thus, the chronic reference dose used in a relatively unrefined risk assessment process is 0.034 mkd (USEPA 2009). These dose levels can be compared to the dose necessary to elicit effects on male mating behavior or mildly increased incidence of thyroid tumors in rats with triadimefon (1800 ppm or approximately 100 mkd in rats), which are used to set the LPEAD in Figure 2. In the guideline multi-generation studies for

triadimefon, 1800 ppm dietary exposures (approximately 100 mkd) in rats produced a number of findings, including liver toxicity and BW effects, as well as effects on male mating behavior, some minor changes in reproductive tissue weights, and minor effects on serum testosterone concentrations. In chronic 2-year exposures in rats, the same dose, 1800 ppm, elicited a slightly higher incidence of follicular adenomas in the rat thyroid concomitantly with histopathological findings in the liver and BW decrements. The NOAEL for these effects in both studies was 300 ppm, which is equivalent to approximately 15 mkd in the rat. Assuming similar uncertainty factors are appropriate for this NOAEL based on possible endocrine-related effects (100×), the chronic reference dose would be approximately 0.15 mkd, which is approximately four-fold higher than the chronic reference dose based on hyperactivity in rodents. Thus, from a risk perspective, neither the effects on male mating behavior (and resultant fecundity) in the multi-generation study nor the rodent thyroid tumors in the rat chronic study would affect the human health risk assessment of triadimefon. For propiconazole, EPA defines the chronic reference dose as 0.1 mkd based on non-neoplastic liver effects seen in a 24-month mouse study with a NOAEL of 10 mkd (USEPA 2014d); this NOAEL is less than 5% of the LPEAD in Figure 3. For myclobutanil, EPA defines the chronic reference dose as 0.025 mkd, based on testicular atrophy in the chronic rat study with a NOAEL of 2.49 mkd (USEPA 2008), approximately 25% of the LPEAD. It is important to note that the exposure assessments used to compare to the LPEAD are largely unrefined and conservative (Methods section), indicating that the margins between the LPEAD and EPA exposure range estimates are likely even greater than the values shown herein; thus, the margins suggested by the approach illustrated in the top panels of Figures 2–4 are likely underestimated. This illustrates the protective nature of the current risk assessment process for PAIs, regardless of the target toxicity.

In this case study, published values from EPA risk assessments, HTS data from the ToxCast/Tox21 programs and ExpoCast exposure estimates were used for comparisons. The Css from a high-throughput toxicokinetic prediction approach was also published by EPA in Scientific Advisory Panel materials from July 2014 (USEPA 2014c). It is understood by stakeholders that the high-throughput toxicokinetic Css values, ExpoCast exposure ranges and even AC50 values from ToxCast are approximations helpful to prioritization, but they are not refined measures of human exposure, hazard or pharmacokinetic/pharmacodynamic parameters for chemicals. For screening, these values are useful in determining relative priority for further endocrine testing. As such, the Css values could be refined via further measurement of the biological variables for each PAI, e.g., fraction unbound in plasma, intrinsic metabolic clearance and glomerular filtration rate, which are involved in estimation of the Css (Wetmore et al. 2012; USEPA 2014c). Similarly, human exposure estimates could be further refined based on current risk assessment approaches to better capture use scenarios and environmental fate, as the estimated values used in this assessment were largely unrefined (see Methods section). These opportunities for refinements to inputs for science-based prioritization provide a mechanism for chemical registrants to supply additional information as needed to correct relative prioritization, noting that these refinements may only be needed when a relative priority ranking indicates a priority that is likely under- or over-estimated.

Regarding whether, in the absence of the EDSP Tier 1 assays, human health-protective prioritization decisions could be made regarding the need for any additional hazard and exposure information

The three triazole fungicides that were used as the source of data for evaluation in the present paper have a rich dataset developed from assessment in HTS assays (ToxCast/Tox21), 40 CFR Part 158 guideline toxicology studies and EDSP Tier 1 assays along with published OSRI that expands the available knowledgebase of toxicity and mechanistic testing for these PAIs. In fact, PAIs represent one of the most studied groups of chemicals in global commerce. Based on this rich dataset, these compounds provide a worthy case study to evaluate the strength and predictive power of high-throughput endocrine screens in combination with high-throughput human exposure predictions to correctly assess the priority of a chemical for EDSP testing.

As discussed earlier, good concordance between HTS assays and the EDSP Tier 1 assay results was observed regarding the absence of ER agonism, ER antagonism, AR agonism and AR antagonism for all three triazoles. Propiconazole had a weak AR antagonism AUC value (0.111) that was matched by consistent effects in the *in vitro* EDSP screens related to this endpoint, but only at high concentrations; the lack of evidence for any AR antagonist effects in the full *in vivo* database with propiconazole confirmed that this weak signal was not translated into effects within the whole organism. Based on the lack of effects in these pathways from the HTS results and the 40 CFR Part 158 guideline toxicology studies, the three triazoles would be of low to no priority for any further testing of direct effects on estrogen or androgen signaling. This lack of direct effects on ER and AR pathway activity was confirmed in the EDSP Tier 1 assays and published OSRI.

Some perturbations of thyroid catabolism signals were observed for the three triazoles in the HTS assays, with triadimefon possibly having a slightly greater signal (based on both UGT1A1 and SULT2A1 induction) than propiconazole or myclobutanil. The HTS data demonstrated a potential upregulation of hepatic catabolism that could affect thyroid hormone homeostasis, as reflected in the mild histologic changes in the thyroid of rats treated with high doses (1800 ppm) of triadimefon over a chronic period (Bomhard & Schilde 1991) and decreased T4 and/or T3 in rats with the same dose (1800 ppm) over shorter exposure periods (Rockett et al. 2006; Wolf et al. 2006; Goetz et al. 2007). Based on the HTS assay data that suggested upregulated markers of Phase I and Phase II metabolism for triadimefon as well as myclobutanil and propiconazole, however, prediction of the presence or degree of *in vivo* changes in thyroid hormone homeostasis or thyroid histology is currently limited without consideration of *in vivo* study information, e.g., from 40 CFR Part 158 guideline toxicology studies, male/female pubertal assays in the EDSP

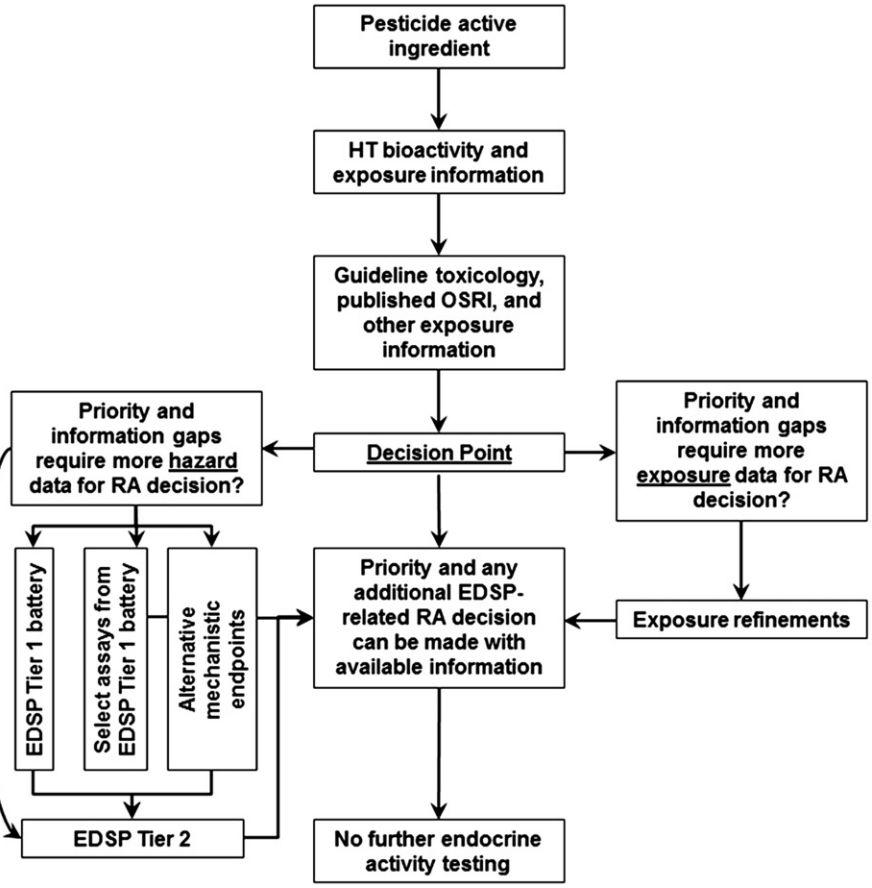

Figure 5. Proposed roadmap for EDSP evaluation of pesticide active ingredients (PAIs). For PAIs, the available high-throughput (HT) hazard and exposure information would be considered first, followed by evaluation of the available guideline toxicology study information before making any priority decisions. Priority decisions could include decisions to collect additional hazard or exposure information, or to make a risk assessment (RA) decision based on adequate available data or suitable margins of separation between predicted exposure and bioactivity.

Tier 1 battery, or alternative mechanistic studies. The HTS dataset indicated that effects on regulation of thyroid hormone catabolism were of weak potency and likely occurred at concentrations equivalent to or in excess of the estimated cytotoxicity limit, in accordance with the *in vivo* database, which suggested for all three triazoles that adaptive metabolic changes (at lower dose levels) and adverse histopathology changes (at higher dose levels) occurred in the livers of rats and mice.

Finally, for steroidogenesis, HTS assays suggested aromatase inhibition for all three triazoles, concordant with aromatase inhibition and steroidogenesis perturbation signals observed in the *in vitro* aromatase inhibition and steroidogenesis assays in the EDSP Tier 1 battery and OSRI. The *in vivo* effects of the three triazoles, however, varied greatly, as already discussed. Propiconazole did not produce clear effects in mammalian species that related to this pathway, including a two-generation reproduction study in rats and pubertal male and female studies (Rockett et al. 2006; Goetz et al. 2007) where no effects on reproductive success were observed. The effects for triadimefon on F1 male mating behavior are likely secondary to systemic toxicity and/or the neuroactivity of triadimefon following increased exposure in the F1 generation. For myclobutanil, because of the presence of histologic findings in the testis and prostate, along with decreased sperm counts, these effects may result from concomitant aromatase inhibition and systemic toxicity.

In Figures 2–4, we present a visual tool for contextualizing HTS indicators of endocrine-related bioactivity with HTS human exposure predictions. Despite the differences between the three triazoles in the eventual *in vivo* outcomes of EDSP Tier 1 testing and the 40 CFR Part 158 guideline toxicity studies, these comparisons in Figures 2–4 show a consistent pattern across all three compounds, namely, that at least three orders of magnitude on a log scale separate the high-throughput predicted exposures and the lowest oral equivalent dose for endocrine-related HTS assay positive results. This wide margin of separation was very similar to the differences between EPA chronic aggregate exposure estimates based on unrefined models, which are generally conservative, and the LPEAD, building confidence that the use of HTS-based prioritization is sufficiently human health protective for prioritization-level decision making related to endocrine activity. Clearly, for compounds with wide margins separating endocrine-related HTS bioactivity assay results and high-throughput human exposure predictions, similar to those demonstrated by the dataset for myclobutanil, propiconazole and triadimefon, there would be little to no priority for further EDSP Tier 1 screening.

An approach for the evaluation of PAIs for endocrine potential is presented in Figure 5. This roadmap enables an

organized, science-based assessment of the potential for endocrine effects in combination with the potential for human exposure. High-throughput bioactivity and exposure information should be considered first; in the future, given further development of integrated, predictive bioactivity models, this step may be comprehensive. For PAIs undergoing EDSP evaluation currently, evaluation of the available high-throughput data for bioactivity and exposure should be followed by examination of corresponding 40 CFR Part 158 guideline toxicology studies, published OSRI and other exposure information to reach a decision point based on the combined assessment of all existing data. Three possible outcomes of the initial decision are (1) more hazard data are needed; (2) more exposure data are needed; or (3) sufficient data are available to both describe the overall prioritization and perform a risk assessment. For most PAIs, a rich bioactivity database is likely to be available, and thus a very limited need for additional hazard data, i.e., EDSP Tier 1 testing or additional exposure information, would be anticipated. Somewhat similar methods for systematic evaluation of endocrine potential, including available bioactivity and exposure information, have been proposed previously to make science-based decisions and reduce animal and resource usage (Willett et al. 2011).

Additionally, the Organization for Economic Co-operation and Development (OECD) has previously developed a conceptual framework (OECD 2012) for interpreting information related to potential endocrine activity from different study types and sources. HTS data, including data from the ToxCast and Tox21 research programs, were not specifically addressed in this OECD framework. Based on the present case study, we assert that HTS assay data, like that available from the ToxCast and Tox21 initiatives, can be considered in a systematic evaluation as conducted herein as prioritization-level information, in concert with human exposure predictions, to make human health-protective prioritization decisions. Furthermore, the HTS bioactivity data and 40 CFR Part 158 guideline toxicology data together comprise a data resource that should be completely reviewed when available to generate the best science-based prioritization decision, thereby optimizing resources used for any additional endocrine activity testing.

Conclusions

For three data-rich triazole fungicides (triadimefon, propiconazole and myclobutanil), the HTS assay dataset was concordant with two other lines of evidence (40 CFR Part 158 guideline toxicology studies and the combined EDSP Tier 1 screens plus published OSRI) in the types of endocrine activity that it predicted. The combined lines of evidence indicated that these three triazoles do not directly act on estrogen, androgen or thyroid systems. *In vitro* evidence of altered steroidogenesis via inhibition of aromatase was observed for all three triazoles, but the degree to which these *in vitro* properties were expressed in mammals *in vivo* differed greatly. Comparison of the endocrine-related HTS bioactivity information with high-throughput human exposure predictions demonstrated the following conclusions:

- Comparison of HTS for endocrine-related bioactivity with high-throughput exposure estimates was useful for prioritization of these data-rich compounds.
- The margins between high-throughput exposure predictions and the endocrine-related HTS bioactivity (converted to oral mkd dose levels) were similar to the margins between unrefined EPA exposure estimates and apical outcomes of *in vivo* studies related to potential endocrine effects (LPEAD). These margins (three to five orders of magnitude) were human health protective and suitable for EDSP screening prioritization decisions.
- Based on the wide margins present between estimates of predicted bioactivity and human exposure, compounds with profiles like those of triadimefon, propiconazole and myclobutanil would have been of low priority for further endocrine screening and testing, had this methodology been available prior to EDSP Tier 1 screening.

Using an EDSP prioritization roadmap for PAIs that is outlined within this manuscript, whereby a prioritization decision would have been made via sequential consideration of HTS for bioactivity and exposure and the 40 CFR Part 158 guideline toxicology studies and other available information (Figure 5), it is unlikely that the EDSP Tier 1 assay results would have been necessary to make a prioritization and risk assessment decision for these three triazoles.

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Declaration of interest

The contents of this manuscript are solely the responsibility of the authors and do not necessarily reflect the views or policies of their employers. All authors are employed by registrants of triazole fungicides. In the current manuscript, interpretations of EDSP Tier 1 studies, OSRI (including published results), and 40 CFR Part 158 guideline studies are based on the data as presented, and as interpreted by the current authors after viewing the wider database for each test substance. Discussion or interpretation of results from individual publications by the authors of those publications is identified, when needed.

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