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J Sanseverino
University of Tennessee-Knoxville

M E. Eldridge

A C. Layton

Terry W. Schultz
University of Tennessee-Knoxville

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Screening of Potentially Hormonally Active Chemicals Using Bioluminescent Yeast Bioreporters

John Sanseverino,*,†,[1] Melanie L. Eldridge,* Alice C. Layton,*,† James P. Easter,* Jason Yarbrough,‡
Terry Wayne Schultz,*,‡ and Gary S. Sayler*,†

*The Center for Environmental Biotechnology; †The Department of Microbiology; and ‡The Department of Comparative Medicine, The University of Tennessee, Knoxville, Tennessee 37996

Saccharomyces cerevisiae bioluminescent bioreporter assays were developed previously to assess a chemical's estrogenic or androgenic disrupting potential. *S. cerevisiae* BLYES, *S. cerevisiae* BLYAS, *S. cerevisiae* BLYR, were used to assess their reproducibility and utility in screening 68, 69, and 71 chemicals for estrogenic, androgenic, and toxic effects, respectively. EC_{50} values were $6.3 \pm 2.4 \times 10^{-10}$M ($n = 18$) and $1.1 \pm 0.5 \times 10^{-8}$M ($n = 13$) for BLYES and BLYAS, using 17β-estradiol and 5α-dihydrotestosterone over concentration ranges of 2.5×10^{-12} through 1.0×10^{-6}M, respectively. Based on analysis of replicate standard curves and comparison to background controls, a set of quantitative rules have been formulated to interpret data and determine if a chemical is potentially hormonally active, toxic, both, or neither. The results demonstrated that these assays are applicable for Tier I chemical screening in Environmental Protection Agency's Endocrine Disruptor Screening and Testing Program as well as for monitoring endocrine-disrupting activity of unknown chemicals in water.

Key Words: Saccharomyces cerevisiae; bioluminescence; estrogens; androgens; biosensing.

A broad survey of our nation's surface waters found widespread presence of 95 organic wastewater contaminants (e.g., Focazio *et al.*, 2008; Kolpin *et al.*, 2002) with coprostanol, cholesterol, N,N-diethyltoluamide, caffeine, triclosan, tri(2-chloroethyl)phosphate, and *p*-nonylphenol as the most prevalent compounds. These compounds may be introduced into surface waters either deliberately (land application), through leaking sewer lines and septic systems, or by incomplete removal from wastewater treatment systems. A wide variety of these chemicals, including pesticides, plasticizers, synthetic hormones and naturally occurring chemicals, possessing steroid-like activity, have been implicated in endocrine disruption in invertebrates and vertebrates (Cooper and Kavlock, 1997; Fang *et al.*, 2000; Folmar *et al.*,

2002; Fossi and Marsili, 2003; Guillette *et al.*, 1999; Kavlock *et al.*, 1996; Ropstad *et al.*, 2006; Sonne *et al.*, 2006; Tyler *et al.*, 1998). Although certain classes of chemicals are known to be endocrine disruptors, the complete scope with regards to the identity and number of chemicals possessing hormonal activity remains unknown. The Environmental Protection Agency, under the auspices of the Food Quality Protection Act of 1996 and the Safe Drinking Water Act of 1996 has developed a screening program for evaluating the potential of chemical substances to induce hormone-related health effects. This screening approach is enormous in scope, with the Environmental Protection Agency (EPA) estimating that 87,000 existing and new chemicals require screening for hormonal activity (EDSTAC, 1998).

To accomplish this task, the EPA proposed a three-part screening protocol to prioritize chemicals for in-depth testing; priority setting, Tier 1 screening, and Tier 2 screening. Priority setting focuses on identifying chemicals that require further testing; that is, excluding chemicals with little or no known hormonal activity and that are generally regarded as safe. The intent of Tier I screening is to rapidly identify chemicals that interact with the estrogen, androgen, and thyroid systems, whereas Tier 2 screenings provide a more in-depth study of how each chemical interacts with each endocrine system. To facilitate Tier I objectives, a high-throughput screening (HTS) mechanism is required for identification of chemicals requiring more in-depth screening. Colorimetric-based yeast bioassays have been used to evaluate the potential for chemicals to cause endocrine-mediated effects. Two widely used receptor/reporter assays for detecting estrogenic and androgenic compounds are the Yeast Estrogen Screen (YES) (Routledge and Sumpter, 1996) and the Yeast Androgen Screen (YAS) (Purvis *et al.*, 1991). These assays have been used extensively to measure endocrine responses to polychlorinated biphenyls (PCBs) and hydroxylated derivatives (Layton *et al.*, 2000; Schultz, 2002; Schultz *et al.*, 1998), polynuclear aromatic hydrocarbons (PAH) (Schultz and Sinks, 2002), pesticides (Sohoni *et al.*, 2001), and other compounds (Schultz *et al.*, 2002) as well as detection of estrogens/androgens in environmental waterways

[1] To whom correspondence should be addressed at The Department of Microbiology, The University of Tennessee, Knoxville, TN 37996. E-mail: jsansev@utk.edu.

(Thomas *et al.*, 2002), aquifers (Conroy *et al.*, 2005), wastewater treatment systems (Layton *et al.*, 2000) and dairy manure (Raman *et al.*, 2004). Additional yeast-based bioreporters have been developed using either a colorimetric detection (Bovee *et al.*, 2004; Gaido *et al.*, 1997; Le Guével and Pakdel, 2001; Rehmann *et al.*, 1999), green fluorescent protein (Bovee *et al.*, 2004, 2007) or the firefly luciferase bioreporter (Bovee *et al.*, 2004; Leskinen *et al.*, 2005; Michelini *et al.*, 2005).

Recently, the *Photorhabdus luminescens lux* operon has been substituted for the *lacZ* gene in the YES assay (*S. cerevisiae* BLYES; Sanseverino *et al.*, 2005) and the YAS assay (*S. cerevisiae* BLYAS; Eldridge *et al.*, 2007). Comparison of these strains to their colorimetric counterparts and proof-of-concept as to their utility has been established (Eldridge *et al.*, 2007; Sanseverino *et al.*, 2005). The purpose of this work was to test strains BLYES and BLYAS against a suite of chemicals with known estrogenic or androgenic activity as identified by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 2002) for validating *in vitro* assays. These chemicals include natural products, pesticides, pharmaceuticals, and steroids, industrial chemical intermediates, plasticizers, and analytical reagents. In addition, specific criteria were developed for data quality evaluation and acceptance.

MATERIALS AND METHODS

Strains and growth media. Estrogen and androgen-inducible strains *S. cerevisiae* BLYES and *S. cerevisiae* BLYAS as well as constitutive *S. cerevisiae* BLYR have been described previously (Eldridge *et al.*, 2007; Sanseverino *et al.*, 2005). *S. cerevisiae* strains harboring plasmids with leucine and uracil selective markers were grown in modified minimal medium without leucine and uracil (YMM leu$^-$, ura$^-$) (Routledge and Sumpter, 1996).

Chemicals. All chemicals, purities, and sources are listed in Table 1. Chemicals were used at the listed purities. High-performance liquid chromatography (HPLC) grade methanol was purchased from Fisher Scientific.

Agonist assay. Strains BLYES, BLYAS, and BLYR were grown in YMM (leu-, ura-) overnight at 30 °C and 200 rpm shaking to an OD_{600} of 1.0. Typically, chemicals were diluted in methanol to stock concentrations of 1, 0.5, and 0.25mM, and then placed on a Beckman F/X Automated Liquid Handling System platform. The robotic system performed 1:2 serial dilutions of each stock concentration (final concentration range of 2.5×10^{-9} through 1.0×10^{-3}M), placing 20 µl of each solution into the appropriate wells of multiple black 96-well Microfluor microtiter plates (Dynex Technologies, Chantilly, VA). Residual methanol was removed by evaporation. Two-hundred microliters of culture were placed into each well of the 96-well plate. For each test assay, a duplicate plate was created using the toxicity control strain BLYR. Bioluminescence was measured every 60 min for 12 h in a Perkin-Elmer Victor2 Multilabel Counter with an integration time of 1 s per well. Positive controls were 17β-estradiol and 5α-dihydrotestosterone (DHT) (concentration range of 2.5×10^{-12} throgh 1.0×10^{-6}M) for the estrogen and androgen assays, respectively. Negative controls included wells with (1) medium + cells and (2) medium + cells + methanol.

Data analysis. For each chemical, bioluminescence (counts per second) versus the log of chemical concentration (M) was plotted generating a sigmoidal

curve for hormonally active compounds. A 50% effective concentration (EC_{50}) value was determined from the midpoint of the linear portion of the sigmoidal dose-response curve. The mean and standard deviation values were calculated from replicate EC_{50} values for each standard to determine the variability between assays. EC_{20} values were determined by calculating the concentration of chemical at 20% above background bioluminescence. Toxic responses (IC_{20}) were determined by calculating the concentration of chemical at 20% less than the background bioluminescence. Toxic equivalency quotients (TEQ) were calculated by dividing the EC_{50} (or EC_{20}) of 17β-estradiol or DHT by the EC_{50} (or EC_{20}) of the test chemical.

RESULTS

Agonist Assay

Methanol was the solvent used to solubilize all chemicals and methanol controls were used in each microtiter plate to monitor background effects. The first criterion for accepting data was to monitor bioluminescence produced in wells containing the cells, medium and solvent (methanol) versus wells that just contained medium and cells. If the methanol:blank bioluminescence ratio was greater than 150% of that for wells with medium and cells alone, then the data for that plate were rejected (data not shown). This was necessary because methanol (including HPLC grade) was shown to carry impurities that influenced EC_{50} measurements (data not shown). Solvent purity was an issue in performing these assays and must be checked regularly. Solvents (especially, those in plastic bottles) may leach impurities that influence the estrogen or androgen response in these strains. Although the ICCVAM report (ICCVAM, 2002) promoted the use of ethanol, the incidence of hormonally active impurities was consistently present (data not shown) necessitating the use of methanol as a solvent.

The negative control, or blank, represents the baseline bioluminescence of the assay. For the methanol blanks, methanol only is added to the wells and is subjected to the same treatment processes as the test chemical, which includes evaporation followed by the addition of 200 µl of culture." Thus, in addition to being a baseline for the assay, it also serves as an instrument control. Any deviations in bioluminescence would indicate potential chemical contamination from the automated liquid handling system, splashing, or some other source of error.

Standard curves (18 points) were included in each microtiter plate for the BLYES and BLYAS assays. The mean and standard deviations of bioluminescence was determined for standard curves for 18 and 13 assays of 17β-estradiol and DHT, respectively (Fig. 1). For each assay, values for minimum and maximum bioluminescence were determined by calculating the mean bioluminescence values from the lower and upper limbs of the standard curve (Table 1). For 17β-estradiol, the lower signal response limit was the mean bioluminescence of the four data points corresponding to 2.5×10^{-12} through 2.5×10^{-11}M (Fig. 1). Likewise, the upper signal response limit of detection was the mean of nine data

TABLE 1
Inventory of Chemicals Used in this Study

Substance	CAS #	Source	Cat. #	Purity	Product Class
Actinomycin D	50-76-0	Sigma	A1410	~98%	Pharmaceutical
Ammonium perchlorate	7790-98-9	Aldrich	208507	99.8%	Industrial
4-Androstenedione	63-05-8	Aldrich	285137	98.0%	Hormone
Apigenin	520-36-5	Sigma	A3145	~95%	Flavenoid
Atrazine	1912-24-9	Supelco	49085	99.9%	Pesticide
Bicalutamide	90357-06-5	TRC	B382000	98.0%	Pharmaceutical
Bisphenol A	80-05-7	Aldrich	13302-7	97.0%	Chemical Intermediate
Butyl benzyl phthalate	85-68-7	Supelco	442503	99.3%	Plasticizer
2-sec-Butylphenol	89-72-5	Aldrich	B99006	98.0%	Pharmaceutical
Clomiphene citrate	50-41-9	Sigma	C6272	100.0%	Pharmaceutical
Corticosterone	50-22-6	Fluka	27840	≥98.5%	Pharmaceutical
Coumestrol	479-13-0	Fluka	27883	≥98%	Natural Product
4-Cumylphenol	599-64-4	Aldrich	C87800	99.0%	Chemical Intermediate
Cycloheximide	66-81-9	Supelco	PS1002	≥95.0%	Pharmaceutical
Cyproterone acetate	427-51-0	Sigma	C3412	98.0%	Pharmaceutical
Daidzein	486-66-8	Sigma	D7802	≥98%	Natural Product
p,p'-DDE	72-55-9	Supelco	49016	99.0%	Pesticide Metabolite
o,p'-DDT	789-02-6	Supelco	49018	97.9%	Pesticide
Dexamethasone	50-02-2	Sigma	D6645	98.0%	Pharmaceutical
Di-n-butyl phthalate	84-74-2	Supelco	PS900	99.5%	Plasticizer
Diethylhexyl phthalate	117-81-7	Supelco	48557	99.0%	Plasticizer
Diethylstilbestrol	56-53-1	Sigma	D4628	≥99%	Pharmaceutical
5α-Dihydrotestosterone	521-18-6	Sigma	A8380	≥98%	Pharmaceutical
17α-Estradiol	57-91-0	Sigma	E8750	≥98%	Hormone
17β-Estradiol	50-28-2	Sigma	E8875	99.0%	Hormone
Estrone	53-16-7	Sigma	E9750	≥99%	Pharmaceutical
17α-Ethynylestradiol	57-63-6	Sigma	E4876	99.4%	Pharmaceutical
Ethyl 4-hydroxybenzoate	120-47-8	Aldrich	111988	99.0%	Pharmaceutical
Fenarimol	60168-88-9	Supelco	PS1073	99.0%	Pesticide
Fenitrothion	122-14-5	Sigma-Supelco	442592		Pesticide
Flavone	525-82-6	Fluka	46370	≥99.0%	Natural Product
Fluoranthene	206-44-0	Supelco	48535	99.2%	Polyaromatic hydrocarbon
Fluoxymesterone	76-43-7	Sigma	F7751	>99%	Pharmaceutical
Flutamide	13311-84-7	Sigma	F9397	≥99%	Pharmaceutical
Formestane	566-48-3	Sigma	F2552	99.6%	Pharmaceutical
Genistein	446-72-0	Sigma	G6649	>98%	Natural Product
Haloperidol	52-86-8	Sigma	H1512	98.0%	Pharmaceutical
Hexestrol	84-16-2	Sigma	H7753	≥98%	Pharmaceutical
Hydrocortisone	50-23-7	BD Biosciences	354203	100.0%	Steroid
17α-Hydroxyprogesterone	68-96-2	Sigma	H5752	≥95%	Hormone
4-Hydroxytamoxifen	68047-06-3	Sigma	H7904	≥98%	Pharmaceutical
Kaempferol	520-18-3	Fluka	60010	≥96%	Natural Product
Kepone	143-50-0	Supelco	49046	98.7%	Pesticide
Ketoconazole	65277-42-1	Sigma	K1003	≥98%	Pharmaceutical
Linuron	330-55-2	Supelco	PS372	98.2%	Pesticide
Medroxyprogesterone acetate	71-58-9	Aldrich	286648	97.0%	Pharmaceutical
Melengestrol acetate	2919-66-6	MP Biomedicals	158952	99.0%	Pharmaceutical
Methoxychlor	72-43-5	Supelco	49054	90.9%	Pesticide
17α-Methyltestosterone	58-18-4	Sigma	M7252	≥98%	Pharmaceutical
Mifepristone	84371-65-3	Sigma	M8046	98.0%	Pharmaceutical
Nilutamide	63612-50-0	Sigma	N8534	100.0%	Pharmaceutical
p-Nonylphenol	104-40-5	Supelco	442873	98.5%	Industrial
Norethynodrel	68-23-5	Sigma	N7253	99.1%	Chemical Intermediate
D(−)-Norgestrel	7997-63-7	Sigma	N2260	99.9%	Pharmaceutical
19-Nortestosterone	434-22-0	Sigma	N-7252	>99%	Hormone
4-tert-Octylphenol	140-66-9	Supelco	442858	99.1%	Chemical Intermediate
Oxazepam	604-75-1	Sigma	O5254	≥99%	Pharmaceutical
Phenobarbital	57-30-7	Sigma	P5178	~95%	Pharmaceutical

TABLE 1—_Continued_

Substance	CAS #	Source	Cat. #	Purity	Product Class
Phenolphthalin	81-90-3	Sigma	P8903	~95%	Analytical Reagent
Pimozide	2062-78-4	Sigma	P1793	≥99%	Pharmaceutical
Procymidon	32809-16-8	Aldrich	36640	≥99.9%	Pesticide
Progesterone	57-83-0	Sigma	P8783	≥98.0%	Pharmaceutical
Propylthiouracil	51-52-5	Fluka	82460	≥99.0%	Pharmaceutical
Sodium azide	26628-22-8	Aldrich	438456	99.0%	Analytical Reagent
Spironolactone	52-01-7	Aldrich	223158	≥99%	Pharmaceutical
Tamoxifen	10540-29-1	Sigma	T5648	≥98%	Pharmaceutical
Testosterone	58-22-0	Sigma	T1500	≥99%	Hormone
Trenbolone	10161-33-8	Sigma	T3925	99.0%	Pharmaceutical
2,4,5-Trichlorophenoxyacetic acid	93-76-5	Supelco	PS45	99.0%	Pesticide
Vinclozolin	50471-44-8	Supelco	PS1049	≥98%	Pesticide
Zearalenone	17924-92-4	Fluka	96093	≥97.5%	Natural Product

points corresponding to 2.5×10^{-9} through 1.0×10^{-6}M. A similar method was used to determine the upper and lower signal response limits for the androgen assay from the DHT standard curve (Table 2). The intraassay variability (%CV) of the EC_{50} values from individual standard curve was 38.1 and 43.6% for the BLYES and BLYAS, respectively. Thus, the range of EC_{50} values for each assay and chemical would be approximately half of one-order of magnitude.

Chemical Testing

A suite of chemicals (Table 1) were used to evaluate the estrogen, androgen, and toxicity responses in BLYES, BLYAS, and BLYR, respectively. EC_{20}, EC_{50}, and IC_{20} values for selected chemicals are highlighted in Tables 3 and 4. In each assay, chemicals that are hormonally active display a sigmoidal curve with lower and upper limbs similar to the standard curve (Fig. 2). Example dose-response curves for 17β-estradiol, 17α-estradiol, 4-_tert_-octylphenol, and mifepristone using strain BLYES are shown in Figure 2A. 17α-Estradiol and 4-_tert_-octylphenol displayed a full sigmoidal dose-response curve and EC_{50} values were 1.1×10^{-8} and 1.4×10^{-7}M, respectively. 4-_tert_-Octylphenol displayed a lower limb and sigmoidal section of the curve but also demonstrated a sharp decrease in bioluminescence at high concentrations (> 1.0×10^{-4}M) indicating chemical toxicity. Mifepristone, although displaying estrogenic activity, did not develop a full sigmoidal curve but rather demonstrated toxicity at concentrations higher than ~5.0×10^{-6}M. Similar dose-response curves were produced using the BLYAS strain (Fig. 2B). DHT and 17β-estradiol produced a full sigmoidal dose-response curve. Mifepristone also displayed androgenic activity but the response reached a plateau at ~1×10^{-5}M.

Toxic effects of chemicals were confirmed with the constitutive bioreporter (BLYR) (Fig. 2C). Toxicity with mifepristone and 4-_tert_-octylphenol was confirmed and IC_{20} values were 1.2×10^{-5} and 2.2×10^{-4}M, respectively (Table 3). For each chemical tested, each assay correctly determined if the chemical was estrogenic, androgenic, toxic, both estrogenic/

androgenic and toxic, or neither (Tables 3 and 4) relative to the data reported in ICCVAM (2002). In addition, it was determined that some chemicals are cross-reactive between both the estrogen-sensing and androgen-sensing reporter strains, for example, 17α-estradiol and cyproterone acetate.
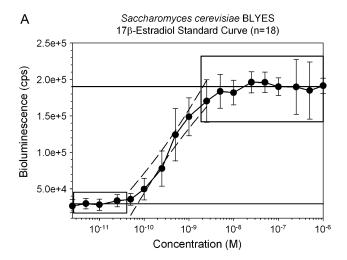
The reproducibility of the standard curves and the range of responses for each test chemical allowed development of quantitative rules to allow automated data collection and interpretation (Fig. 3). The proposed rules define if data from each assay are acceptable and if an EC_{50} can be determined. Each hormonally active chemical with no associated toxicity produced a complete sigmoidal curve with minimum and maximum bioluminescent responses within the standard deviation of the standard curves.

If it is determined that a complete sigmoidal curve is present, then the EC_{50} is calculated by determining the chemical concentration at the midpoint of the exponential portion of the sigmoidal curve. Alternatively, if the curve is incomplete, then an EC_{20} concentration for induction is calculated by determining the concentration necessary to produce bioluminescence at 20% above background bioluminescence

DISCUSSION

Yeast-based _in vitro_ estrogen and androgen screens have been firmly established as a means for rapidly identifying chemicals with potential endocrine-disrupting activity. An endocrine disruptor is an exogenous substance that causes adverse health effects in an organism or its offspring by way of alteration in the function of the endocrine system. As such endocrine disruption is a mechanism leading to a variety of adverse health effects, most of which are considered as reproductive or developmental toxicities (OECD, 2002). The yeast reporters used in this study utilize human receptor protein and response elements to activate transcription of a reporter gene (Zacharewski, 1997). Thus, it is important to realize that yeast-based systems cannot explicitly identify
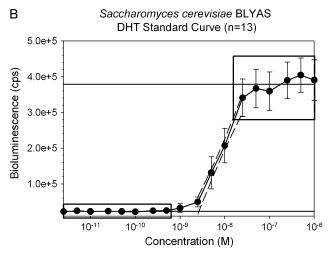
FIG. 1. (A) *Saccharomyces cerevisiae* BLYES 17β-estradiol standard curve. This curve was compiled from 18 individual assays. (B) *S. cerevisiae* BLYAS DHT standard curve. This curve was compiled from 13 individual assays. Error bars represent the standard deviation of bioluminescence for each data point. Dashed lines represent the 95% confidence intervals for each assay.

endocrine disruptors because yeast do not have an endocrine system.

The complex nature of reproductive and developmental effects suggests that *in vivo* tests are necessary to detect endocrine disruption. However, as pathways leading to

TABLE 2
Summary of Bioluminescent Yeast Bioreporter Assay Characteristics

Assay	Chemical standard	EC_{50} (M)	Upper limit of detection (M)	Lower limit of detection (M)
BLYES	17β-Estradiol	$6.3 \pm 2.4 \times 10^{-10}$	5.0×10^{-9}	2.5×10^{-11}
BLYAS	DHT	$1.1 \pm 0.5 \times 10^{-8}$	5.0×10^{-8}	1.0×10^{-9}

reproductive and development effects are elucidated, the binding to members of the nuclear hormone receptor superfamily and subsequent activation or repression of transcription has been shown to be one critical step, which can lead to adverse reproductive effects. This criticality reflects the fact that such nuclear receptors act as ligand-dependent transcription factors, which mediate the effects of hormones to regulate the expression of specific genes, which in turn affect reproduction and development.

In vitro tests, especially recombinant receptor transcription assays using yeast cells with response element-regulated reporter genes, have been proven to be effective in quantifying receptor binding and are commonly used in first stage screening of chemicals for endocrine activity. The first generation colorimetric-based assays, in particular those using β-galactosidase (Purvis *et al.*, 1991; Routledge and Sumpter, 1996), are well-established and reliable reporter gene assays. One significant advantage of bioluminescence assays compared with colorimetric assays is speed. Quantifiable bioluminescence using BLYES and BLYAS was observed in 60 min with maximum bioluminescence observed in 3–4 h (Eldridge *et al.*, 2007; Sanseverino *et al.*, 2005). In contrast, the colorimetric assay required 3 days before a response was measured and for target compounds or environmental samples with low estrogenicity, 5 days of incubation were required for detection of the estrogenic response (Layton *et al.*, 2000, 2002; Raman *et al.*, 2004; Schultz *et al.*, 1998). The BLYES and BLYAS assays are comparable to the colorimetric and Luc-based yeast bioreporters reported previously (Table 5). The interassay variability for the EC_{50} values listed in Table 5 are $3.8 \pm 1.9 \times 10^{-10}$ M, $1.1 \pm 1.1 \times 10^{-8}$ M, and $2.1 \pm 2.8 \times 10^{-8}$ M, for 17β-estradiol, DHT, and testosterone, respectively. This suggests that the BLYES and BLYAS assays are consistent with previously published yeast-based reporter assays (Table 5). The 40–50% variability of the EC_{50} values reaffirms the suggestion that no single assay should be used to determine an absolute EC_{50} value but rather as a first step in estimating the hormonal activity of a chemical (Beresford *et al.*, 2000).

Yeast-based systems have proven their reliability for chemical screening however they do have certain limitations. Beresford *et al.* (2000) outlined various factors that can influence responses in the colorimetric assay including incubation time and temperature, cell inoculum, metabolic inactivation of the compound, and submaximal responses. These same issues are present in the bioluminescent yeast assay as well (Sanseverino *et al.*, 2005). Yeast-based assays, whether they are colorimetric or bioluminescent, are only one method for determining a compound's hormonal activity. Detailed characterization of hormonal activity should be performed *in vivo*. Our intent for these bioluminescent assays is to serve as a screening tool for identification of compounds that require further characterization.

The dose-response curves were performed over a range of six-orders of magnitude ($\sim 10^{-9}$–10^{-3}M). This range was

TABLE 3
Summary of Responses of All Chemicals Tested with the BLYES Assay

Chemical name	Estrogenic activity		Relative potency		Toxicity IC$_{20}$
	EC$_{20}$ (M)	EC$_{50}$ (M)	TEQ$_{20}$	TEQ$_{50}$	
Actinomycin D	—	—	—	—	1.86E−04
Ammonium perchlorate	3.26E−04	—	4.29E−07	—	1.00E−04
4-Androstenedione	—	—	—	—	na[a]
Apigenin	nr[b]	—	nr	—	na
Atrazine	—	—	—	—	5.3E−04
Bisphenol A	6.20E−04	—	2.26E−07	—	na
Butyl benzyl phthalate	2.15E−05	4.68E−05	6.21E−06	—	na
2-sec-Butylphenol	3.70E−04	na	3.78E−07	—	na
Clomiphene citrate	—	na	—	—	na
Corticosterone	8.06E−5	1.97E−07	1.71E−06	3.2E−03	3.29E−06
Coumestrol	5.00E−08	3.30E−08	2.80E−03	1.91E−02	na
4-Cumylphenol	8.40E−07	1.26E−06	1.67E−04	5.00E−04	7.23E−05
Cycloheximide	5.00E−05	4.50E−05	2.80E−06	1.40E−05	na
Cyproterone acetate	7.30E−07	1.57E−05	1.92E−04	4.01E−05	8.47E−04
Daidzein	—	na	—	—	na
p,p'-DDE	4.26E−05	9.20E−05	3.29E−06	6.85E−06	na
o,p'-DDT	7.68E−04	—	1.82E−07	—	na
Dexamethasone	1.00E−05	6.30E−05	1.40E−05	1.00E−05	na
Di-n-butyl phthalate	0	na	—	—	na
Diethylhexyl phthalate	0	na	—	—	
Diethylstilbestrol	1.30E−10	6.42E−10	1.08E+00	9.81E−01	na
5α-Dihydrotestosterone	1.15E−06	3.71E−06	1.22E−04	1.70E−04	9.86E−04
17α-Estradiol	1.50E−09	1.10E−08	9.33E−02	5.73E02	na
17β-Estradiol	1.4E−10	6.3E−10	1	1	na
Estrone	2.10E−08	6.40E−09	6.67E−03	9.84E−02	na
17α-Ethynylestradiol	na	2.50E−11	na	2.52E+1	na
Ethyl 4-OH-benzoate	6.40E−07	1.40E−06	2.19E−04	4.50E−04	na
Fenarimol	6.10E−05	—	2.30E−06	—	8.18E−05
Fenitrothion	1.67E−04	—	8.38E−07	—	na
Flavone	4.60E−05	—	3.04E−06	—	9.11E−05
Fluoranthene	—	—	—	—	2.08E−05
Fluoxymesterone	—	—	—	—	na
Flutamide	—	—	—	—	na
Formestane	—	—	—	—	na
Genistein	6.4E−07	3.86E−06	2.19E−04	3.39E−01	na
Haloperidol	—	—	—	—	3.84E−05
Hexestrol	1.34E−10	8.70E−10			na
Hydrocortisone	—	—	—	—	na
17α-OH-progesterone	—	—	—	—	na
4-Hydroxytamoxifen	8.33E−10	1.86E−09	1.68E−01	3.39E−01	5.00E−08
Kaempferol	—	—	—	—	7.93E−06
Kepone	—	—	—	—	9.12E−06
Ketoconazole	7.4E−04	—	1.89E−07	—	na
Linuron	nr	—	—	—	na
Medroxyprogesterone acetate	4.00E−04	—	3.5E−07	—	na
Methoxychlor	—	—	—	—	3.63E−05
17α-Methyltestosterone	3.56E−06	7.20E−06	3.93E−05	8.75E−05	8.00E−04
Mifepristone	1.40E−06	—	—	—	1.18E−05
Nilutamide	—	—	—	—	4.19E−04
p-Nonylphenol	4.23E−08	1.64E−07	3.31E−03	3.84E−03	9.56E−04
Norethynodrel	1.70E−05	1.38E−04	8.24E−06	4.57E−06	4.28E−05
D(−)-Norgestrel	nr	—	nr	—	na
19-Nortestosterone	nr	—	nr	—	na
4-tert-Octophenol	3.30E−08	1.41E−07	4.24E−03	4.47E−03	2.24E−04
Oxazepam	—	—	—	—	na
Phenobarbital	3.87E−04	—	3.62E−07	—	na

TABLE 3—*Continued*

| Chemical name | Estrogenic activity | | Relative potency | | Toxicity |
	EC$_{20}$ (M)	EC$_{50}$ (M)	TEQ$_{20}$	TEQ$_{50}$	IC$_{20}$
Phenolphthalin	—	—	—	—	na
Pimozide	5.00E−07	8.89E−07	2.80E−04	7.09E−04	na
Procymidon	3.90E−06	4.07E−06	3.59E−05	1.55E−04	1.17E−04
Progesterone	2.09E−04	—	6.70E−07	—	9.99E−05
Propylthiouracil	7.42E−04	—	1.89E−07	—	na
Sodium azide	—	—	—	—	9.50E−04
Tamoxifen	nr	—	—	—	na
Testosterone	1.30E−05	2.23E−05	1.08E−05	2.83E−05	4.19E−04
Trenbolone	2.69E−05	4.50E−05	5.20E−06	1.40E−05	na
2,4,5-Trichlorophenoxy acetic acid	—	—	—	—	na
Vinclozolin	—	—	—	—	na
Zearalenone	7.90E−07	1.90E−06	1.77E−04	3.32E−04	1.79E−05

[a]na, not applicable.
[b]nr, not reportable.

chosen to characterize the assay's ability to measure extreme concentrations. In the BLYES assay, several compounds had an in complete dose-response curve and an EC$_{20}$ of 10^{-4}M (fenitrothion [1.67×10^{-4}M], ketoconaide [7.40×10^{-4}M], medroxyprogesterone acetate [4.0×10^{-4}M], phenobarbital [3.87×10^{-4}M], propyl thiouracil [7.42×10^{-4}M], 2-s butyl phenol [3.7×10^{-4}M]) (Table 3). Further *in vivo* testing will be required to determine if these concentrations are physiologically relevant. All data should be interpreted in the broader scale of the science.

A significant issue present in the use of yeast-based bioreporter assays performed in microtiter plates is chemical solubility. In this study, chemicals that would not dissolve in methanol were not evaluated (dibenzo[*a,h*]anthracene, 12-*O*-tetradecanoylphorbol-13-acetate, l-thyroxine, and reserpine). Thus, an alternative protocol for screening highly hydrophobic compounds and reducing binding to microtiter plates is required. In previous work, Layton *et al.* (2002) used *S. cerevisiae* YES to compare ^{14}C-labeled 4-chlorobiphenyl (4-CB) added to plastic microtiter plates and glass vials before and after medium addition. The standard operating procedure (SOP) as described in this paper was to add test compound in solvent (10 μl) to the microtiter plate and let the solvent evaporate before adding medium. In a modified operating procedure (MOP), medium was added first followed by test compound in 2 μl of solvent. Bioavailability (3–26%) of ^{14}C-labeled 4-CB was highest using the MOP in glass vials. This was approximately double the availability using the SOP. Beresford *et al.* (2000) also compared adding butyl benzyl phthalate and 4-nonylphenol directly to the medium versus evaporation of the ethanol solvent followed by medium addition. They found that although their colorimetric assay was more sensitive with solvent addition to the medium, the relative potency of each test chemical was the same in both methods relative to 17β-estradiol. Adding hydrophobic chem-

icals directly to yeast medium may increase bioavailability, however, nonspecific solvent effects on bioluminescence and potential yeast toxicity needs to be monitored. The constitutive strain BLYR served this purpose. In the context of a HTS, the user needs to be aware of the solubility of each test compound. Compounds with extremely low solubility may have to use a modified procedure such as the one described by Layton *et al.* (2002).

When used as a Tier 1 screening tool, the battery of BLYES, BLYAS, and BLYR provides the quantitative data needed to proceed through the various steps in the workflow outlined in Figure 4. Based on Figure 4, there are five outcomes from the bioluminescent yeast bioreporter screening:

• *Chemical is presumptive hormonally active.* These are chemicals that display bioluminescence, produce a full sigmoidal dose-response curve and have no toxicity. Chemicals tested that fall into this category include: butyl benzyl phthalate, dexamethasone, diethylstilbestrol, *p*-nonylphenol, 4-hydroxytamoxifen (Table 3), and 4-androstenedione, clomiphene citrate, cyproterone acetate, hydrocortisone, and trenbolone (Table 4).

• *Chemical is presumptive hormonally active and displays toxicity.* This group produced limited bioluminescence (no sigmoidal dose-response curve). Bioluminescence was hampered due to a chemical's toxicity at higher concentrations. An EC$_{50}$ value cannot be calculated from this data. This group included fenarimol, flavone, mifepristone, progesterone (Table 3) and fenitrothion (Table 4).

• *Chemical has presumptive hormonal activity but an EC$_{50}$ cannot be calculated.* This group of chemicals produced an incomplete dose-response curve. In most cases, this was due to the concentration range tested was not broad enough to capture the full sigmoidal dose-response curve. However, chemicals with limited solubility may also display incomplete dose-response

TABLE 4
Summary of Responses of All Chemicals Tests with the BLYAS Assay

Chemical name	Androgenic activity		Relative potency	
	EC_{20} (M)	EC_{50} (M)	TEQ_{20}	TEQ_{50}
Ammonium perchlorate	—[a]	—	—	—
4-Androstenedione	5.40E−08	2.03E−07	8.98E−02	5.32E−02
Atrazine	—	—	—	—
Bicalutamide	4.11E−04	—	1.18E−05	—
Bisphenol A	—	—	—	—
Butyl benzyl phthalate	—	—	—	—
2-sec-Butylphenol	—	—	—	—
Clomiphene citrate	6.35E−07	2.14E−06	7.64E−03	5.05E−03
Corticosterone	—	—	—	—
Coumestrol	—	—	—	—
4-Cumylphenol	—	—	—	—
Cycloheximide	—	—	—	—
Cyproterone acetate	4.51E−07	9.63E−07	1.08E−02	1.12E−02
Daidzein	—	—	—	—
p,p'-DDE	—	—	—	—
o,p'-DDT	—	—	—	—
Dexamethasone	—	—	—	—
Di-n-butyl phthalate	—	—	—	—
Diethylhexyl phthalate	—	—	—	—
Diethylstilbestrol	—	—	—	—
5α-Dihydrotestosterone	4.85E−09	1.08E−08	1	1
17α-Estradiol	—	—	—	—
17β-Estradiol	1.33E−05	4.19E−05	3.65E−04	2.58E−04
Estrone	—	—	—	—
17α-Ethynylestradiol	—	—	—	—
Ethyl 4-hydroxybenzoate	—	—	—	—
Fenarimol	—	—	—	—
Fenitrothion	5.59E−06	—	8.68E−04	—
Flavone	—	—	—	—
Fluoranthene	—	—	—	—
Fluoxymesterone	4.20E−08	1.26E−07	1.15E−01	8.57E−02
Flutamide	—	—	—	—
Formestane	1.18E−05	3.39E−05	4.11E−04	3.19E−04
Haloperidol	—	—	—	—
Hexestrol	—	—	—	—
Hydrocortisone	8.28E−06	2.18E−05	5.86E−04	4.95E−04
17α-Hydroxyprogesterone	2.43E−08	4.92E−08	2.00E−01	2.20E−01
4-Hydroxytamoxifen	—	—	—	—
Kaempferol	1.79E−05	3.69E−05	2.71E−04	2.93E−04
Kepone	—	—	—	—
Ketoconazole	—	—	—	—
Linuron	1.72E−07	1.99E−06	2.82E−02	5.43E−03
Medroxyprogesterone acetate	1.20E−06	4.80E−06	4.04E−03	2.25E−03
Melengestrol acetate	—	—	—	—
Methoxychlor	1.34E−06	3.26E−06	3.62E−03	3.31E−03
17α-Methyltestosterone	8.06E−09	1.45E−08	6.02E−01	7.45E−01
Mifepristone	2.44E−06	—	1.99E−03	—
Nilutamide	—	—	—	—
p-Nonylphenol	—	—	—	—
Norethynodrel	4.66E−07	3.03E−06	1.04E−02	3.56E−03
D(−)-Norgestrel	5.10E−08	2.79E−07	9.51E−02	3.87E−02
19-Nortestosterone	3.99E−08	8.20E−08	1.22E−01	1.32E−01
4-tert-Octophenol	—	—	—	—
Oxazepam	—	—	—	—
Phenobarbital	—	—	—	—

TABLE 4—Continued

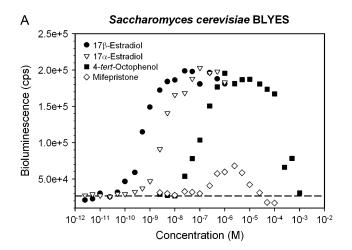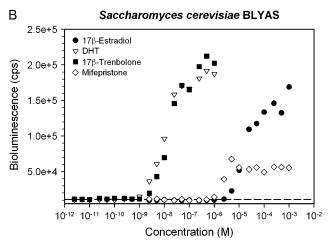| Chemical name	Androgenic activity		Relative potency	
	EC_{20} (M)	EC_{50} (M)	TEQ_{20}	TEQ_{50}
Phenolphthalin	—	—	—	—
Pimozide	—	—	—	—
Procymidon	1.93E−04	2.91E−04	2.51E−05	3.71E−05
Progesterone	—	—	—	—
Propylthiouracil	1.13E−06	6.71E−07	4.29E−03	1.61E−02
Sodium azide	—	—	—	—
Spironolactone	2.72E−06	6.56E−06	1.78E−03	1.65E−03
Tamoxifen	—	—	—	—
Testosterone	1.93E−09	8.31E−09	2.51	1.30
Trenbolone	1.27E−08	2.66E−08	3.82E−01	4.06E−01
2,4,5-Trichlorophenoxyacetic acid	—	—	—	—
Vinclozolin	—	—	—	—
Zearalenone	—	—	—	—

[a] No response.

curves. Examples of this type of response include: bisphenol A, ketoconazole, phenobarbital (Table 3), and bicalutamide (Table 4). Generally, an EC_{50} cannot be determined from this data, but an EC_{20} can be calculated. The EC_{20} is defined as the concentration at which bioluminescence is increased by 20%.

• *Chemical is toxic*. These are chemicals that cause a decrease in bioluminescence in the constitutive strain BLYR. Chemicals that fall into this category include: atrazine, haloperidol, kepone, methoxychlor, and sodium azide (Table 3). An IC_{50} cannot be determined from this data, but an IC_{20} can be calculated.

• *Chemical is not hormonally active and not toxic*. There is no increase in bioluminescence in the BLYES and BLYAS strains and no decrease in bioluminescence in the BLYR strain. An example is phenolphthalin (Table 3).

In the present study, the results of chemical screening using BLYES and BLYAS for 68 substances with known estrogen and androgen responses, (ICCVAM, 2002) are reported. Although the majority of responses measured using the yeast-based bioreporter assays were consistent with the ICCVAM framework, there were some inconsistencies in chemical responses in comparison with the ICCVAM (2002) report. Of particular concern in using these assays would be false negatives, that is, chemicals that do not induce a response in yeast assays but in fact are endocrine disruptors. False negatives can arise from a number of factors including high hydrophobicity and poor solubility, toxicity, and metabolic activation of the chemical by mammalian systems. Estrogenic compounds which showed potential false negatives includes four compounds listed in ICVAMM as weak estrogen agonists but for which no activity was detected in the BLYES assay (clomiphene citrate, kaempferol, kepone, and methoxychlor). These require further *in vivo* testing. Clomiphene citrate was reported previously as an estrogenic agent (9.97×10^{-6}M)
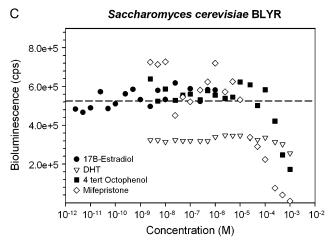
Proposed Rules for *S. cerevisiae* BLYES and BLYAS Data Acceptance

1. Bioluminescence produced in the methanol control must be less than 150% of the bioluminescence produced in the assay blank.

2. Minimum bioluminescence in the 17β-estradiol and DHT standard curve must be approximately 29,600 ± 9,400 and 23,500 ± 6,000 counts per second, respectively.

3. Maximum bioluminescence in the 17β-estradiol and DHT standard curve must be approximately 190,200 ± 14,000 and 379,000 ± 88,500 counts per second, respectively. 17β-Estradiol and DHT standard curve plots and test chemical plots should be sigmoidal.

4. Values for the upper and lower limbs for each test chemical must be within the standard deviation of the respective standard curves.

5. Chemical toxicity, measured by the constitutive bioluminescent strain *S. cerevisiae* BLYR, must be absent in the sigmoidal part of the standard curve.

FIG. 3. Proposed rules for data acceptance from the *Saccharomyces cerevisiae* BLYES, *S. cerevisiae* BLYAS, and *S. cerevisiae* BLYR assays.

An advantage and disadvantage of yeast-based assays is their inability to metabolically activate a target compound. Previous studies have demonstrated that for certain chemicals including PCBs and PAHs, (Layton *et al.*, 2002; Schultz, 2002; Schultz and Sinks, 2002), the hydroxylated metabolites, and not the parent compound, induce the estrogenic response. The short incubation times (3 h) for these bioluminescent assays may not be sufficient to activate certain chemicals (e.g., methoxychlor, diadzein). For example, methoxychlor is metabolized to 2,2-bis(*p*-hydroyphenol)-1,1,1-trichloroethane which is estrogenic. In the BLYES assay, methoxychlor and diadzein were nonresponsive. Beresford *et al.* (2000) reported an estrogenic response to methoxychlor after 3–5 days of incubation in a colorimetric assay. These researchers suggested that when metabolites are known and available, they should be tested alongside the parent compound. In yeast assays with short incubation periods, incubation of the chemical with liver extracts or P450 systems may be considered to activate the chemical.

Several chemicals differed from the ICCVAM report in that they demonstrated weak potential estrogenic activity. These include three androgen agonists (cyproterone acetate [EC_{20} 4.51 × 10^{-7}M], medroxyprogesterone acetate [EC_{20} 1.20 × 10^{-6}M], spironolactone [EC_{20} 2.72 × 10^{-6}M]) and one androgen antagonist (procymidon; EC_{20} 1.93 × 10^{-4}). These compounds displayed cross-reactivity at relatively high doses in yeast estrogen assays. Gaido *et al.* (1997) and Beresford *et al.* (2000) note that these dosages are unrealistic and subsequently not physiologically important. Twenty chemicals in the BLYES assay had an EC_{20} in the range of 10^{-4}–10^{-5}M. Further *in vivo* testing would be required to determine if these are physiologically relevant concentrations.

Integrated testing strategies (Blaauboer *et al.*, 1999) make use of all the available relevant and reliable information in a tiered approach of increasing biological complexity in the hazard and risk assessment process. Significant to this approach are *in vitro* tests and screens, including cell cultures.

FIG. 2. Dose-response and toxicity curves for select chemicals generated using (A) *Saccharomyces cerevisiae* BLYES, (B) *S. cerevisiae* BLYAS, and (C) *S. cerevisiae* BLYR. Dashed line represents the average background bioluminescence of the bioreporter.

using a different yeast assay (Gaido *et al.*, 1997). Differences in assays that report weak activity versus no activity may be protocol dependent (e.g., incubation time, species/cultures employed).

TABLE 5
Comparison of EC_{50} Values Derived from Yeast-Based Estrogen and Androgen Assays

	Test compound	EC_{50} (M)	References
Estrogen assay			
Saccharomyces cerevisiae BLYES	17β-Estradiol	6.3×10^{-10}	This study
S. cerevisiae BMAEREluc	17β-Estradiol	5.0×10^{-10}	Leskinen *et al.*, 2005
S. cerevisiae YES	17β-Estradiol	4.0×10^{-10}	Sanseverino *et al.*, 2005
S. cerevisiae yEGFP-S2	17β-Estradiol	4.0×10^{-10}	Bovee *et al.*, 2004
S. cerevisiae Luc-S2	17β-Estradiol	2.0×10^{-10}	Bovee *et al.*, 2004
S. cerevisiae βGal-S2	17β-Estradiol	2.0×10^{-10}	Bovee *et al.*, 2004
S. cerevisiae BJ3505 (pYEPKB1 and pYRPE2)	17β-Estradiol	2.3×10^{-10}	Gaido *et al.*, 1997
Yeast strain (unidentified)	17β-Estradiol	1.5×10^{-9}	Rehmann *et al.*, 1999
S. cerevisiae BJ-ECZ (hER-lacZ)	17β-Estradiol	7.0×10^{-10}	Le Guével and Pakdel, 2001
Androgen assay			
S. cerevisiae BLYAS	DHT	1.1×10^{-8}	This study
S. cerevisiae BLYAS	Testosterone	7.5×10^{-9}	This study
S. cerevisiae BMAAREluc	DHT	5.5×10^{-9}	Leskinen *et al.*, 2005
S. cerevisiae BMA64-1A (pYipLuc)	Testosterone	1.0×10^{-8}	Michelini *et al.*, 2005
S. cerevisiae YAS	DHT	3.5×10^{-9}	Eldridge *et al.*, 2007
S. cerevisiae YAS	Testosterone	4.7×10^{-9}	Eldridge *et al.*, 2007
S. cerevisiae YPH500	DHT	3.5×10^{-9}	Gaido *et al.*, 1997
S. cerevisiae YPH500	Testosterone	4.7×10^{-9}	Gaido *et al.*, 1997
S. cerevisiae (p406-ARE2-*CYC1*-yEGFP)	Testosterone	7.6×10^{-8}	Bovee *et al.*, 2007
S. cerevisiae (p406-ARE2-*CYC1*-yEGFP)	DHT	3.3×10^{-8}	Bovee *et al.*, 2007

Increasingly, the most useful of such cell culture systems are ones which are optimized to recognize and quantify a unifying feature such as activation of a regulatory sequence key to a toxic pathway. Such systems have high specific applicability and when linked to a reporter system have the potential to be used in high-throughput testing. The BLYES, BLYAS, and BLYR battery of assays form such an *in vitro* screen.

Structure-activity relationships (SARs) are also part of integrated testing strategies (Blaauboer *et al.*, 1999) and at minimum can p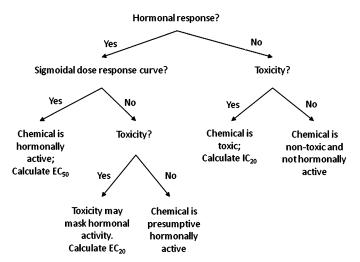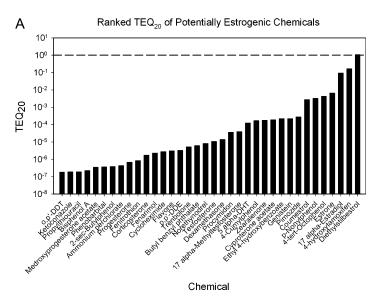rovide guidance on chemical testing. In this study the majority of chemicals tested agreed with the predicted hormonal binding responses with estrogens, some known pharmaceuticals, flavenoids, phenolic industrial chemicals and plasticizers inducing BLYES (Fig. 5A). Likewise, androgenic inducing chemicals included the expected natural and synthetic androgens, and the pesticides linuron and methoxychlor (Fig. 5B). Several responses were detected toward nontarget pharmaceuticals including the reactions of BLYAS to the thyroid pharmaceutical, propylthiouracil, BLYES to the antipsychotic pharmaceutical, pimozide and cross reactions of BLYES and BLYAS to natural and synthetic estrogens and androgens. However, chemicals may also emerge from this study and other studies that do not follow typical SAR for endocrine disruption. For instance, the nonsteroidal and non-phenolic compounds ammonium perchlorate and cycloheximide both induced BLYES. The fact that these chemicals reacted with either BLYES or BLYAS but not both implies a certain level of specificity for that receptor. Other chemicals not tested in this study but warranting further investigation by these reporter strains include arsenic and cadmium which have been implicated in endocrine disruption (e.g., Bodwell *et al.*, 2006; Henson and Chedrese, 2004; Stoica *et al.*, 2000).

FIG. 4. Decision tree for determining if a chemical is potentially hormonally active, toxic, both, or neither.

Conclusions

The purpose of Tier I screening methods is to rapidly identify chemicals that interact with the estrogen, androgen, and thyroid systems. Chemicals identified in Tier I are moved to more detailed studies in Tier II. HTS is required to rapidly categorize the thousands of chemicals in production. Yeast-based
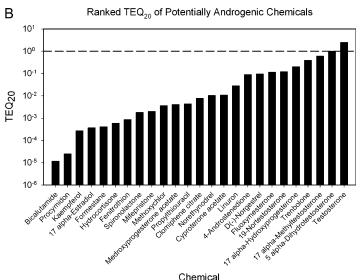
A   Ranked TEQ$_{20}$ of Potentially Estrogenic Chemicals

B   Ranked TEQ$_{20}$ of Potentially Androgenic Chemicals

FIG. 5. TEQ$_{20}$ of chemicals which exhibit potentially estrogenic (A) and androgenic (B) activity. TEQ$_{20}$ is calculated by dividing the EC$_{20}$ of the standard by the EC$_{20}$ of the test compound. The dashed line represents the relative TEQ$_{20}$ of one for 17β-estradiol and DHT.

assays for screening estrogens and androgens fill this need. There are several applications for bioluminescence-based, *lux*-based in particular, bioreporter strains for facilitating Tier I screening for potentially endocrine-disrupting activity. Uses of the assays offer the following:

● High-throughput. Automation of chemical, medium, and cell distribution to microtiter plates was demonstrated with this study. Further, with proper robotics, transfer to a luminescence plate reader is possible.

● Data. These bioreporters can be used as qualitative or quantitative assays. When used as described, EC$_{20}$, EC$_{50}$, and dose-response curves can be generated. This allows ranking of chemicals based on potency relative to standards reducing subjective interpretation of the data.

● Speed. Bioluminescence detection is very sensitive relative to colorimetric assays hence data can be collected in a short period of time; three hours for the bioluminescent assays. Data can be downloaded into a spreadsheet and analyzed by computer algorithm for interpretation.

● Autonomy. Exogenous reagents are not necessary for reporter signal development which reduces costs and manipulations.

It is well documented that pharmaceuticals and personal care products as well as other organic pollutants that cause endocrine-disrupting activity are present in our nation's waste streams and waterways (e.g., Focazio *et al.*, 2008; Kolpin *et al.*, 2002; Owens *et al.*, 2007; Zheng *et al.*, 2008). These assays provide a rapid means of assessing if a water sample has

activity before conducting expensive analytical methodology. This activity can be conducted in the laboratory via water collection and spotting microtiter plates as described. An alternative is to conduct real-time online monitoring by integrating these bioluminescent bioreporters with integrated circuitry equipped with photodetectors (Bolton *et al.*, 2002; Islam *et al.*, 2007; Nivens *et al.*, 2004; Simpson *et al.*, 2001; Vijayaraghavan *et al.*, 2007).

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