Comparison of Five in Vitro Bioassays to Measure Estrogenic Activity in Environmental Waters

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Received January 11, 2010. Revised manuscript received April 8, 2010. Accepted April 12, 2010.

Bioassays are well established in the pharmaceutical industry and single compound analysis, but there is still uncertainty about their usefulness in environmental monitoring. We compared the responses of five bioassays designed to measure estrogenic activity (the yeast estrogen screen, ER-CALUX, MELN, T47D-KBluc, and E-SCREEN assays) and chemical analysis on extracts from four different water sources (groundwater, raw sewage, treated sewage, and river water). All five bioassays displayed similar trends and there was good agreement with analytical chemistry results. The data from the ER-CALUX and E-SCREEN bioassays were robust and predictable, and well-correlated with predictions from chemical analysis. The T47D-KBluc appeared likewise promising, but with a more limited sample size it was less compelling. The YES assay was less sensitive than the other assays by an order of magnitude, which resulted in a larger number of nondetects. The MELN assay was less predictable, although the possibility that this was due to laboratory-specific difficulties cannot be discounted. With standardized bioassay data analysis and consistency of operating protocols, bioanalytical tools are a promising advance in the development of a tiered approach to environmental water quality monitoring.

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Introduction

The presence of contaminants in the aquatic environment is a key challenge facing humanity (*1*). In past decades, the field of endocrine disruption has generated much concern over the potential of environmental contaminants to disrupt endocrine functions in exposed wildlife and human populations. Endocrine disrupting compounds (EDCs) that can mimic or interfere with the functions of estrogens have been extensively studied as estrogens regulate a variety of biological functions in vertebrates such as growth, metabolism, cell growth and proliferation, cell function and differentiation, sexual development and behavior, and development of the immune system in both sexes (*2, 3*). Estrogenic chemicals in the environment have been implicated with a variety of reproductive and physiological abnormalities in wildlife (*2, 4, 5*), and while it is still not entirely clear whether exposure to environmental levels of EDCs currently affects human populations (*2*) there is a need to monitor levels of estrogenicity in the aquatic environment.

In vitro bioassays are now recognized as potent monitoring tools in a tiered approach as they detect chemical contaminants based on their biological action. As the specific chemical composition of an environmental sample is often unknown and mixture interactions cannot always be inferred from the concentrations of the individual components, bioassays are important tools to examine the presence of and integrate the biological activity in complex mixtures of EDCs (*6*).

There is, however, uncertainty about the reliability of bioassays in environmental monitoring. Bioassay results may be affected by other unrelated toxic effects from complex mixtures, and there have been questions about their variability, reliability, and robustness, particularly in dealing with highly polluted samples. There are significant issues with interlaboratory variability due to poor standardization of bioassay techniques (*7*), and there is a perception that bioassays (with a biological detector) are too different from chemical techniques (with a physical detector) to allow comparison between the two methods.

The objectives of this study were to assess the efficacy of commonly used bioassays to detect and quantify estrogenic activity in environmental samples and their ability to complement chemical analysis. This is a critical validation step toward integrating bioassays into risk assessment frameworks that are currently developed for single chemicals. Five in vitro bioassays were selected for evaluation after a comprehensive review of the literature (*8*): the yeast estrogen screen (YES) (*9*), a yeast-based reporter gene assay; the ER-CALUX (*10*), the MELN (*11*), and the T47D-KBluc (*12*) assays, which are mammalian-based reporter gene assays; and the E-SCREEN (*13*) assays, based on proliferation of estrogen-dependent breast cancer cells. Simultaneous chemical analysis was also performed on all samples for comparison. Each of these bioassays has strength and limitations inherent to its design (reviewed in refs *8, 14, and 15)* and the mechanism of estrogen action (described in detail in ref *16*) it monitors.

Experimental Section

Chemicals. The following model compounds were tested in this study: the hormones 17β-Estradiol ("E2", 98% pure, Sigma), estrone ("E1", >99% pure, Aldrich), and estriol ("E3", USP grade, Sigma); the pharmaceuticals 17α-ethynylestradiol ("EE2", 98% pure, Aldrich) and tamoxifen (96% pure, Aldrich); the industrial compounds 4-nonylphenol ("4-NP", technical grade, Aldrich), 4-*tert*-octylphenol ("4tOP", 97% pure, Ald-

10.1021/es903899d
Published on Web 04/27/2010

TABLE 1. Combined Chemical Data (Weighted Averages ± SD)[a]

description	E2(μg/L)	E1(μg/L)	E3(μg/L)	EE2(μg/L)	4tOP(μg/L)	4-NP(μg/L)	BA(μg/L)	BBP(μg/L)
groundwater 1 (shallow aquifer)	<0.005	<0.005	<0.005	<0.005	0.583 ± 0.454	0.271 ± 0.219	<0.025	<5
groundwater 2 (deep aquifer)	<0.005	<0.005	<0.005	<0.005	<0.025	<0.125	<0.025	<5
raw sewage 1	0.016 ± 0.011	0.017 ± 0.012	0.181 ± 0.009	<0.013	2.62 ± 2.53	6.33 ± 1.15	0.433 ± 0.029	<12.5
raw sewage 2	0.024 ± 0.011	0.083 ± 0.021	0.159 ± 0.057	<0.013	0.400 ± 0.087	3.64 ± 5.52	0.633 ± 0.126	<12.5
treated sewage 1	<0.005	0.023 ± 0.021	<0.005	<0.005	0.500 ± 0.278	1.12 ± 0.076	0.050 ± 0.000	<5
treated sewage 2	<0.005	<0.005	<0.005	<0.005	0.233 ± 0.104	0.338 ± 0.239	<0.025	<5
river 1 (large river)	<0.005	<0.005	<0.005	<0.005	0.483 ± 0.029	0.221 ± 0.146	<0.025	<5
river 2 (small stream)	<0.005	0.007 ± 0.007	<0.005	<0.005	0.367 ± 0.153	5.27 ± 4.97	<0.025	<5
groundwater 1 (spiked)	0.025 ± 0.005	0.075 ± 0.022	0.018 ± 0.013	<0.005	5.80 ± 5.10	94.1 ± 9.08	57.4 ± 5.50	720 ± 51.5
groundwater 2 (spiked)	0.014 ± 0.004	0.061 ± 0.005	0.042 ± 0.003	0.008 ± 0.004	0.300 ± 0.100	90.7 ± 11.6	53.2 ± 4.30	775 ± 103
raw sewage 1 (spiked)	0.033 ± 0.008	0.277 ± 0.014	0.197 ± 0.010	<0.013	7.28 ± 7.35	20.7 ± 2.03	47.2 ± 2.24	88.7 ± 52.1
raw sewage 2 (spiked)	0.043 ± 0.010	0.134 ± 0.001	0.279 ± 0.065	<0.013	0.450 ± 0.100	14.8 ± 0.763	46.1 ± 2.77	71.5 ± 10.1
treated sewage 1 (spiked)	0.027 ± 0.005	0.138 ± 0.003	0.055 ± 0.005	<0.005	4.20 ± 5.47	27.8 ± 4.48	46.4 ± 2.87	332 ± 74.5
treated sewage 2 (spiked)	0.007 ± 0.005	<0.005	<0.005	<0.005	0.817 ± 0.679	12.2 ± 1.02	18.5 ± 1.75	373 ± 97.3
river 1 (spiked)	0.010 ± 0.001	0.070 ± 0.007	0.040 ± 0.005	0.006 ± 0.004	1.22 ± 0.321	38.7 ± 1.93	49.6 ± 3.90	341 ± 45.8
river 2 (spiked)	0.008 ± 0.003	0.088 ± 0.005	0.045 ± 0.005	0.008 ± 0.003	0.383 ± 0.126	51.5 ± 2.52	48.2 ± 3.88	551 ± 58.4

[a] n = three fully independent samples, analysed in two different laboratories. Four of the chemicals monitored were never detected: tamoxifen (<0.1 μg/L), endosulfan (<10 μg/L), dieldrin (<10 μg/L), and *p,p'*-DDT (<50 μg/L). Abbreviations used: E2 = 17β-estradiol, E1 = estrone, E3 = estriol, EE2 = 17α-ethynylestradiol, 4tOP = 4-*t*-octylphenol, 4-NP = 4-nonylphenol, BA = bisphenol A; BBP = benzyl butyl phthalate.

rich), bisphenol A ("BA", >99% pure, Aldrich), benzyl butyl phthalate ("BBP", 98% pure, Aldrich) and tetrabromobisphenol A (97% pure, Aldrich); the pesticides dieldrin (99.1% pure, Supelco), α- and β-endosulfan (99.5% pure each, Supelco) and *p,p'*-DDT (99.3% pure, Supelco); and the natural phytoestrogen genistein (>98%, Sigma). The estrogenic potencies of these chemicals relative to 17β-estradiol are presented in Supporting Information (SI) Table S1.

Environmental Samples. Samples from four environmentally relevant compartments were tested: groundwater, raw sewage, treated sewage and river water. All samples were collected in Brisbane (Australia) in methanol-rinsed glass bottles and immediately acidified to pH 2. Six replicate samples were taken at each site, half of which were immediately spiked after pH adjustment with a standard mix of estrogenic chemicals as positive controls (E1, E2, E3, EE2, BA, NP, and BBP; predissolved in ethanol) or with a vehicle control (ethanol; in all cases the final ethanol concentration was 0.08%). The samples were brought back to the laboratory and extracted as described below. The concentration of the spiked compounds in the final extract (tested in the bioassay) determined by chemical analysis is presented in Table 1.

Sample Extraction. Samples were processed immediately upon return to the laboratory. Raw sewage samples were first centrifuged at 4 °C and 2000*g* for 12 min to remove large particulate matter. All samples were then extracted using a standard extraction protocol (*17*) with minor modifications. In brief, samples were filtered with AP20 filters (Millipore) under gentle vacuum, and passed by vacuum through Oasis HLB reversed-phase solid-phase extraction cartridges (Waters Corp). Cartridges were dried on the manifold for 1−2 h (until dry), wrapped in aluminum foil and stored at 4 °C until elution (maximum 10 days). The cartridges were eluted with 5 mL of methanol followed by 5 mL of 1:1 acetone:hexane. The solvent was evaporated to dryness under gentle nitrogen stream, and the extract reconstituted in 500 μL ethanol. Samples were split into 12 40 μL aliquots in MaxRecovery vials (Waters Corp), evaporated to dryness, and flooded with argon. The samples were then shipped to all participating laboratories for analysis.

Bioassays. Five bioassays were performed in seven distinct laboratories: 2× YES, 1× ER-CALUX, 1× MELN, 1× T47D-KBluc, and 2× E-SCREEN assays.

The YES assay was performed in two different laboratories, each using its own previously established and validated protocols. The protocols all follow the standard method for the YES assay (*18*). In brief, yeast cells are distributed in a 96-well plate and exposed to the sample in culture medium for 3 days. The yellow chromogenic substrate CPRG (chlorophenol red-β-D-galactopyranoside) is added and transformed to a red product by β-galactosidase which is measured by spectrophotometer at 540 nm.

The ER-CALUX assay was performed in only one laboratory using a standard method previously described (*10*). Briefly, cells are seeded into 96-well plates two days prior to induction. A day later, the medium is changed to steroid-free medium. On the day of induction, the medium is changed again and replaced by steroid-free medium and the sample. After 24 h of exposure, cells are lysed, luciferin is added to the incubation medium and luciferase activity is measured by luminescence.

The MELN assay was performed in only one laboratory using a previously described protocol (*19*). In brief, cells are seeded into a 96-well plate two days prior to induction. A day later, the medium is changed to steroid-free medium. On the day of induction, the medium is changed again and replaced by steroid-free medium with the sample. After 16−24 h of exposure, cells are lysed, luciferin is added to the incubation medium and luciferase activity is measured by luminescence.

The T47D-KBluc was performed by only one laboratory following a previously published protocol (*12*). In brief, cells are withdrawn into steroid-free medium for 1 week prior to being seeded into a 96-well plate for 24 h, after which they are exposed to the sample for a further 24 h. At the end of the incubation period, cells are lysed and luciferase activity measured by luminescence. Only one

of the three independent replicates for each sample was analyzed in this assay.

The E-SCREEN assay was performed in two different laboratories, each using its own previously established method (*20–22*). Briefly, MCF-7BOS breast cancer cells (a gift of Dr Ana Soto, Tufts University, Boston, MA) are seeded in a 96-well plate in steroid-free medium. After 24 h, the medium is exchanged for fresh steroid-free medium with the sample. After 5 days of exposure, the number of live cells in each well is determined using a standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

Wide Spectrum Chemical Screening. Gas chromatography/mass spectrometry (GC/MS) wide spectrum screening was used to measure E2, E1, E3, TMX, α-endosulfan, β-endosulfan, dieldrin, *p,p'*-DDT, 4tOP, 4-NP, BA, and BBP. The analysis was performed as previously described (*22*).

Targeted Chemical Analysis. Analysis of E1, E2, EE2, E3, 4-NP, 4tOP, and BA was done by GC/MS after solid-phase extraction and silylation of the analytes (*23*). Analysis of BBP, TBBPA, and TMX was done by GC/MS after solid-phase extraction. Analysis of *p,p'*-DDT, dieldrin and endosulfan was done by gas chromatography/electron capture detection (GC/ECD) after liquid−liquid extraction following the procedure described in the German standard method DIN 38407, part 2 (*24*). Analysis of genistein was done by liquid chromatography coupled to tandem mass spectrometry by an electrospray interface (HPLC-ESI-MS-MS).

Data Analysis. Chemical data from the screening and targeted analysis were combined using weighted averages to give more influence to the more precise data (Table 1).

All measurements in the bioassays were expressed as estradiol equivalents (EEq). The EC50 values of the bioassay response (whether galactosidase or luciferase activity or cell proliferation) derived from a 17β-estradiol standard curve (in ng/well) was divided by the EC50 value obtained from a sample dilution series (in L equivalent/well) to obtain the EEq (in ng/L). All data were log-transformed to be compatible with statistical assumptions of normality and homogeneity of variances. Samples below the quantification limit (nondetects) were dealt with separately depending on purpose: for graphical representations and summary of averages and SD, values below the quantification limit of the bioassay were assigned a value of half the quantification limit; while the full data set including values below quantification limit was used for survival (Kaplan−Meier) analysis (Figure 2).

To evaluate the performance of the bioassays irrespective of the type of sample, a survival analysis (Kaplan−Meier analysis) was carried out using GraphPad Prism 5 (GraphPad Software Inc.). This type of analysis allows the statistical comparison of right-censored data sets, and thus the left-censored data from all bioassays in this study were transformed to right-censored data using the following equation:

$x_t = (1)/(\log(x)) + 4$, where x_t is the transformed data and x the original data.

Finally predicted estrogenic activity (Figure 3) was calculated as Pred = ∑(RP × conc), where RP is the relative potency (SI Table S1) and conc is the concentration as determined by analytical chemistry (Table 1). Where available, the specific relative potency obtained in this project was used, otherwise the average of the relative potencies for that bioassay type published in the literature was used (SI Table S1).

Results and Discussion

Chemical Analysis. Chemical data is presented in Table 1.

Bioassays—Model Compounds. Estrogenicity of the individual compounds tested is presented in SI Table S1 (results obtained from this study are highlighted with an asterisk). Overall the estrogenic activity was similar to that previously reported with the exception of 4tOP and 4NP, which were more potent estrogen mimics in the YES assay than previously reported by 1−2 orders of magnitude (SI Table S1). Tetrabromobisphenol A and 4tOP were cytotoxic in the YES and ER-CALUX assays, respectively.

Bioassays—Environmental Samples. All bioassays displayed similar trends in estrogenic activity among the environmental samples, but with different amplitudes (Figure 1). Of the nonspiked environmental samples, the raw sewage samples displayed the highest estrogenic activity in all bioassays (Figure 1, left). Sewage treatment resulted in >88 to >99% removal of the estrogenic activity, depending on the bioassay. Low estrogenic activity was detected in the river samples with some of the bioassays, while estrogenicity in groundwater samples was below the quantification limit of most bioassays except the ER-CALUX and the E-SCREEN assays from the second laboratory (Figure 1). These results are comparable to those reported in previous studies for similar water types (reviewed in ref *14*). Addition of the spike resulted in a predictable increase in estrogenic activity for all sample types in all bioassays with the notable exception of raw sewage in the MELN assay, where addition of the spike caused an unexpected decrease in estrogenic activity (Figure 1C, right).

The YES assays had the highest method quantification limit (MQL; 3.5 and 5 ng/L) while the other bioassays had much lower MQLs: 0.27 ng/L for the MELN assay, 0.2 ng/L for the KBluc and both E-SCREEN assays and 0.1 ng/L for the ER-CALUX. The high MQL of the YES assays meant that activity was not detected in any of the nonspiked samples except the most polluted (i.e., raw sewage; Figure 1A) and the YES assays had the highest proportion of samples below the MQL (40%). Conversely, the low MQL of the ER-CALUX bioassay allowed detection of estrogenic activity in almost all samples, with only 3% of nondetects (Figure 1B). The MELN assay, the KBluc, and the two E-SCREEN assays reported 22−29% of nondetects.

The high proportion of nondetects in most data sets made statistical correlations difficult. When nondetects were assigned a value of half the quantification limit, the coefficient of correlation between all bioassay data sets was high (R^2 > 0.75 for all pairwise comparisons). Although this clearly highlights the good agreement between the different bioassays in identifying samples with high vs low activity, it is biased by assigning an artificial value to nondetect samples. The correlation analysis using the censored data set (i.e., excluding the nondetects altogether) indicated less agreement between assays (average R^2 values: YES = 0.32, ER-CALUX = 0.67, MELN = 0.19, T47D-KBluc = 0.58, and E-SCREEN = 0.64), although of course this ignores a significant portion of the data that would support good agreement.

Comparison of Bioassays. To accurately compare the bioassays and overcome the issue of nondetects, all estrogenic activity data generated from analysis of environmental samples as well as the synthetic samples from model compounds (SI Table S1) were combined and a Kaplan−Meier survival analysis carried out on the entire data set (Figure 2). The analysis shows that the bioassays generate remarkably similar responses, particularly among samples with low- to midestrogenicity (from 0.2 to 20 ng/L, Figure 2C). There were no significant differences between the survival curves of any of the assays (pairwise comparisons, log-rank Mantel-Cox test, $p > 0.05$) with the exception of the MELN survival curve, which was significantly different from all but the YES assay curve ($p < 0.05$, except for MELN vs YES assay where $p = 0.09$).

The results with the MELN assay appear skewed to the left of the survival curve (Figure 2C), suggesting that estrogenicity determined by the MELN was consistently lower than those of the other bioassays with the methodology

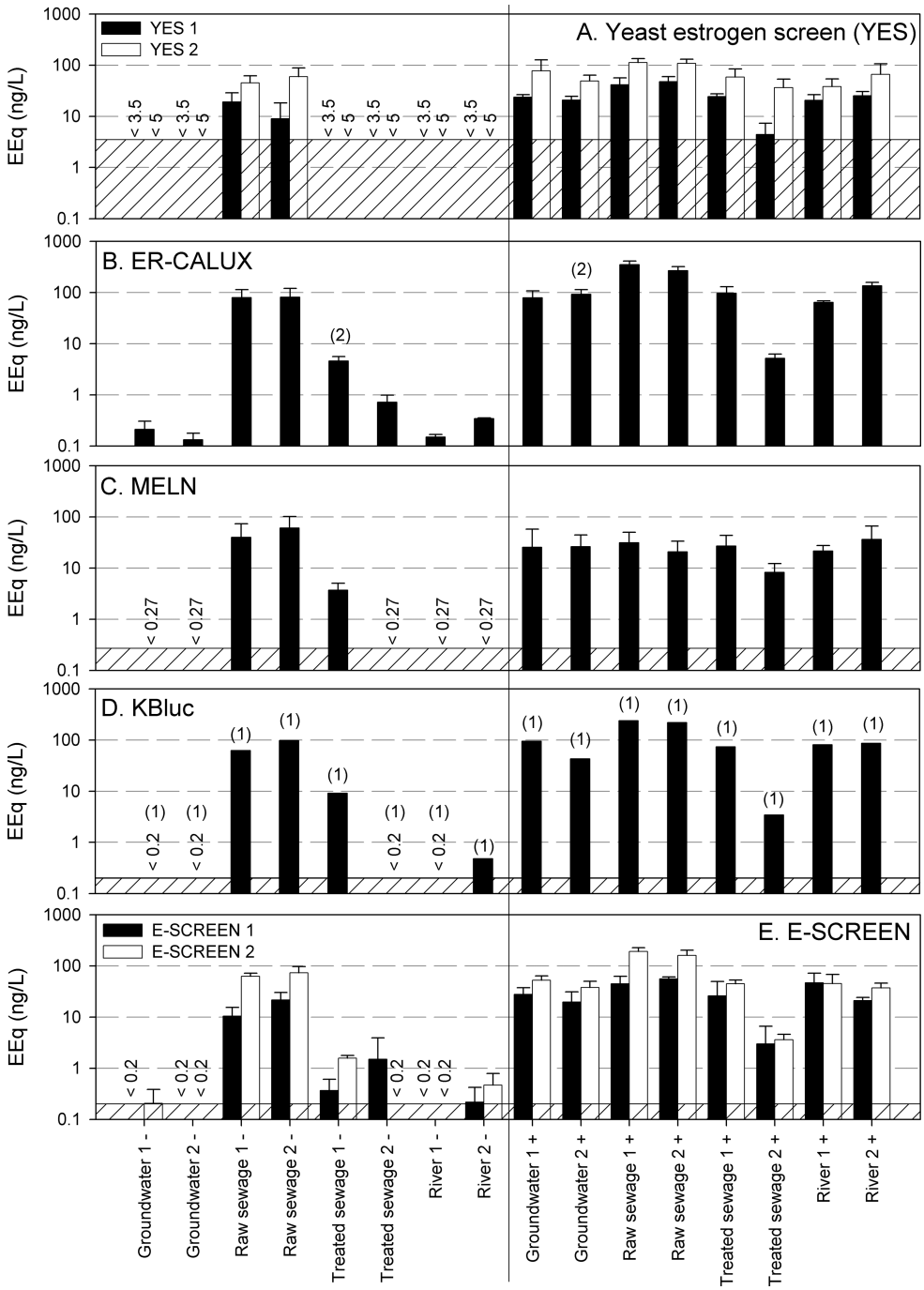

FIGURE 1. Estrogenicity (expressed as estradiol equivalents, EEq) in the environmental samples as determined by each bioassay. Left panel, nonspiked samples; Right panel, spiked samples. Notes: *n* = three independent samples unless otherwise indicated by a number in brackets above the bars. The cross-hatched shaded area indicates data below the method quantification limit (MQL). Error bars represent standard deviations. Data include nondetects, set at half the quantification limit.

practices in this group of laboratories. The YES assay data set was the most heavily censored (46.4 and 45.2% for the YES assays in the first and second laboratory, respectively), a reflection of its comparatively high MQL. Conversely, the ER-CALUX data set had the lowest censorship with only 10% of samples below the MQL.

There were no significant differences in the data set between laboratories for either the YES or the E-SCREEN assays (Figure 2A and B; $p > 0.05$), suggesting that differences between laboratories can be maintained to a minimum with appropriate standardization and quality control.

Bioassays vs Chemistry. By multiplying the concentration of each chemical as determined by standard chemical methods (Table 1) with the relative potency for each individual compound (SI Table S1), it is possible to calculate a predicted estrogenicity for each sample. This predicted estrogenicity based on chemical analysis can then be compared to the estrogenicity measured by bioassays (Figure 3). Discrepancy between predicted and measured estrogenicity can indicate the presence of estrogenic compounds other than those measured by chemical analysis, nonadditive mixture interactions, poor bioassay performance due to matrix interference, or the presence of potent antiestrogens. A matching predicted and measured activity indicates that estrogenicity measured in the bioassay can be accurately predicted from chemical analysis. Of particular interest to

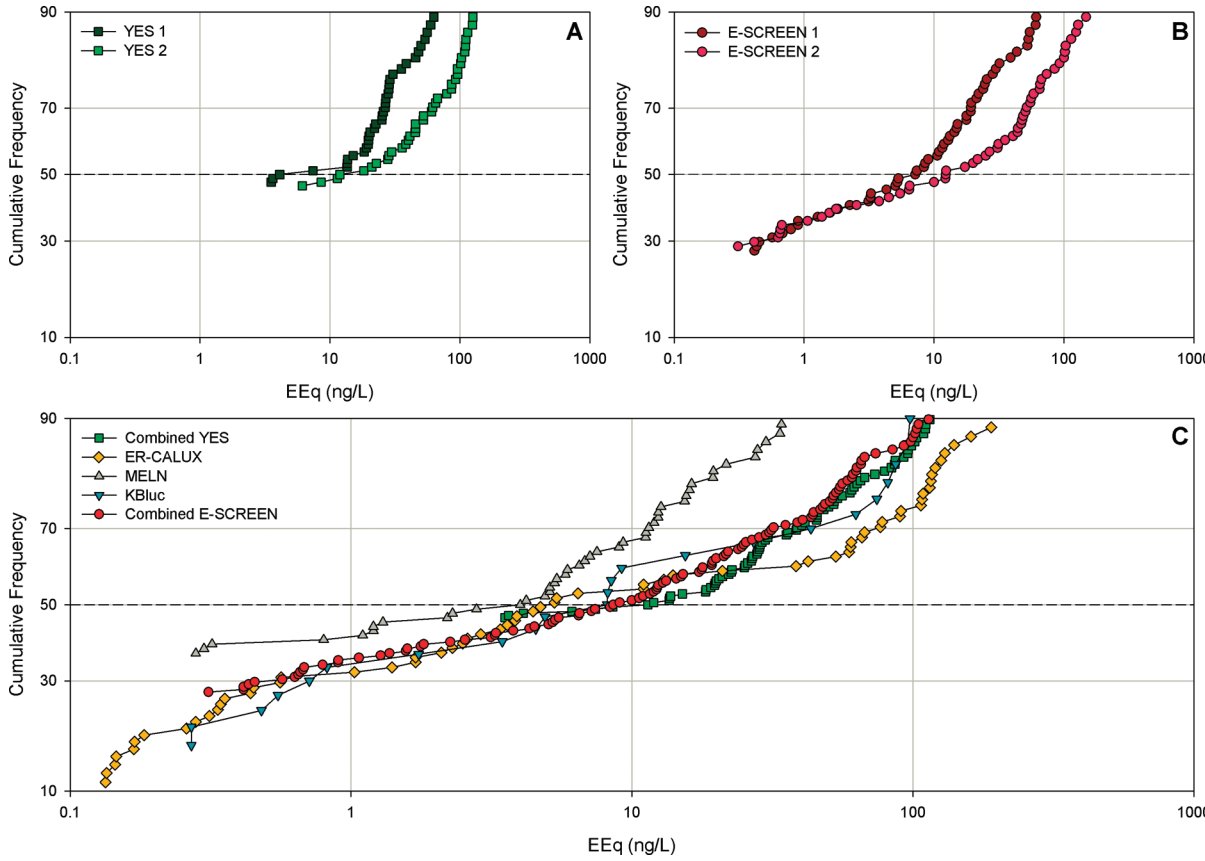

FIGURE 2. Survival graph for bioassay data. (A) YES assays 1 and 2; (B) E-SCREEN assays 1 and 2; (C) All bioassays.

confirm the predictability of the bioassays are the spiked samples, where most of the activity should come from the spiked compounds.

Overall there was a good agreement between the predicted and measured estrogenicity in the ER-CALUX, the second YES assay and both E-SCREEN assays (most spiked samples fall closely on the isometric line in Figure 3) with average predicted/measured ratio with spiked samples of 1.02, 1.27, 1.46, and 1.03, respectively. The MELN assay and the YES assay performed in the first laboratory seemed to slightly underestimate the estrogenic activity, while the KBluc assay overestimated it, with an average predicted/measured ratio with spiked samples of 2.18, 3.32, and 0.61, respectively. The YES assay has previously been reported to underestimate estrogenic activity, possibly due to a high sensitivity to antiestrogenic compounds (*25*). Antiestrogenicity did not appear to affect the other bioassays however, so it is unclear at this stage.

Previous studies have reported both good (*6, 26*) and poor (*27, 28*) agreement between predicted and measured estrogenic activity in sewage and surface water samples. A discrepancy between the two can generally be put down to an incomplete chemical characterization of complex samples (such as raw sewage) or uncertainties in the accuracy of the data used in the model (*27*).

Overall assessment. In vitro bioassays can detect chemicals based on their biological activity and determine the total estrogenic content of a given sample, and thus play a major role as initial screening tools for environmental samples (first-pass screening). This can help prioritize further investigation using in vivo methods and/or chemical identification of active substances. In other words, a significant response in an in vitro bioassay could be used as a trigger for further investigation using in vivo test models, while a negative response would suggest the scope of these financially and ethically expensive investigations can be reduced. Additionally, bioassays can detect potential estrogenic activity from unexpected/unknown EDCs not included in the chemical screening, thus providing further confidence in the monitoring program.

While there were differences in the amplitude of the response, the data trends were very similar between the different assays. There were subtle differences in intensity of estrogenicity between the bioassays that could be due to the mechanism of action of the estrogenic chemicals (reviewed in (*16*)). For example, the reporter gene assays (YES, ER-CALUX, MELN, and KBluc assays) detect receptor-mediated genomic events, while cell proliferation in the E-SCREEN assay would also detect nongenomic estrogenic effects (e.g., via cell surface receptors). Other differences could be due to receptor isomer-specific responses.

A word of caution against generalization is appropriate here. Only two of the five assays tested in this study were replicated in two independent laboratories (YES and E-SCREEN bioassays), and as such this study provides only a limited measure of interlaboratory variability. It is also difficult to determine from our results alone whether the differences observed with the MELN assay for example are assay- or laboratory-specific.

Ultimately, each assay has advantages and limitations. Some limitations apply to all in vitro assays, such as the inability to detect compounds that are pro-estrogens (compounds that are converted to estrogens in vivo) (*15*) or to conclusively identify the causative chemical without chemical analysis. The ER-CALUX and E-SCREEN assays tested in this study successfully measured estrogenicity in environmental water samples, even at very low concentrations. The estrogenic activity measured in these bioassays could be related to predicted estrogenic activity based on chemical analysis, suggesting that either of these bioassays could be used as initial screening tools to detect estrogenicity in environmental water samples. The KBluc assay also appeared to perform

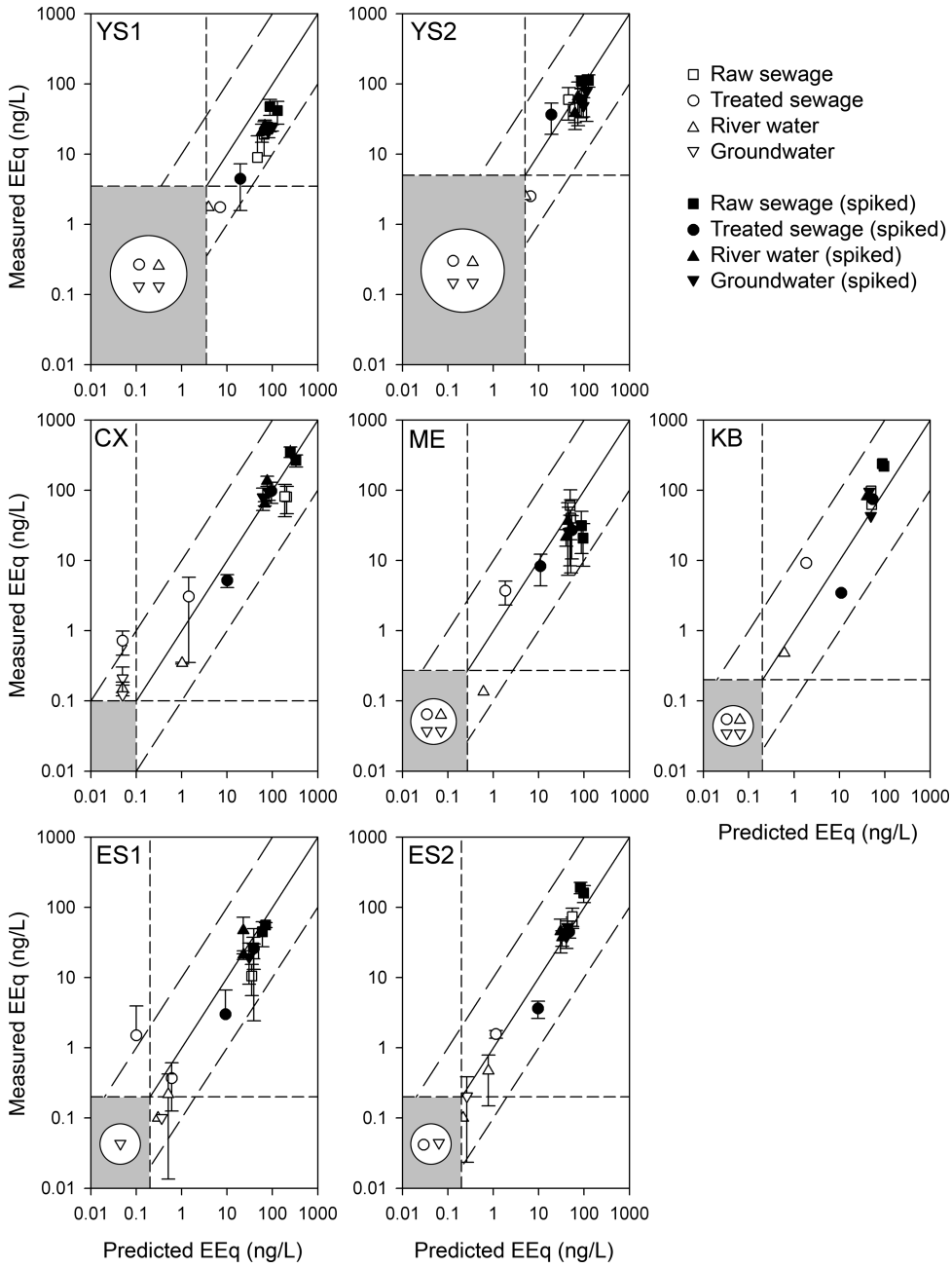

FIGURE 3. Comparison of estrogenic activity (EEq) measured in vitro against predicted from chemical analysis. The circle at the bottom left (in the shaded area below the quantification limit) indicates the number and type of samples in this area. Notes: Each symbol represents the average of three fully independent samples. The dashed horizontal and vertical lines represent the bioassay quantification limit (MQL). The solid line represents the isometric line. YS1 = yeast estrogen screen 1, YS2 = yeast estrogen screen 2, CX = ER-CALUX, ME = MELN, KB = KBluc, ES1 = E-SCREEN 1, ES2 = E-SCREEN 2.

well, and in fact was very similar to the ER-CALUX, but the data set obtained in this study was more limited for that bioassay than for the others, and any conclusion based on the KBluc assay must therefore be appraised critically. The YES assay performed well with highly polluted samples but its relatively high quantification limit meant that it was unable to quantify low-level estrogenicity in less polluted samples such as groundwater, river water and even treated sewage effluent. With synthetic samples, the performance of the YES assay was also significantly affected by the presence of octylphenol (SI Table S1) and cell toxicity appears to be an issue with concentrated environmental aqueous samples (*29*). Furthermore, the yeast cell wall may impede active and passive transport of chemicals to the intracellular space, thus increasing the risk of false negatives (*30*). The MELN assay tested in this study also performed well, correctly identifying low and high estrogenic activity, however it seemed to underestimate estrogenic activity in many of the samples (Figure 1−3). It is possible that laboratory-specific issues could have impacted on the MELN results presented here and independent confirmation of the reported trends is required.

Table 2 provides a matrix summarizing the performance of the bioassays tested in this study and their identified strengths and limitations. The table highlights that each bioassay has its advantages and limitations, and the notion of "fit-for-purpose" is critical in determining what bioassay to use. Ultimately, any of the five bioassays tested may be suitable for testing estrogenic activity in environmental

TABLE 2. Summary of the Strength and Weaknesses and Overall Performance of the Bioassays Tested in This Study[a]

assay	YES	ER-CALUX	MELN	KBluc[a]	E-SCREEN
performance					
environmental samples EEq[b]	+	+++	+	(++)	++
likeness to other assays[c]	++	+++	−	+++	+++
coefficient of variation (CoV)[d]	+++	+++	+	NA	++
method quantification limit (MQL)[e]	−	+++	+++	+++	+++
predicted vs measured comparison[f]	+	+++	+	(++)	++
overall performance in this study[g]	+1/2	++3/4	+1/4	(++1/2)	++1/4
strengths and weaknesses					
analysis of model compounds	+++	+++	++	(+++)	+++
analysis of environmental samples	−[h]	+++	+	(++)	+++
ease of use	++	+	+	+[i]	+
simple training	++	−	−	−[i]	−
low maintenance and consumables cost	+++	−	+	+	+
free access to cell line for nonprofit use	+++	−	+++	++	+++
sensitivity	−	+++	++	++	++
robustness	−[j]	++	++	++	++
reproducibility	++	+++	+	++	++
maturity (widespread use)	+++	++	+	+	+++
high-throughput screening	+++	+++	+++	+++	+++
quick results	++	++	++	++	−

[a] Notes: "-", below average; "+", fair; "++", good; "+++", excellent; NA, data not available. [a] Values in brackets indicate a lower precision due to less samples being analyzed in the KBluc bioassay. [b] Environmental samples estrogenic activity, data in Figure 1. [c] Comparison with other bioassays irrespective of sample source, data in Figure 2. [d] Coefficient of variation, calculated from all data (not shown). [e] Method quantification limit, data in Figure 1 (dashed area). [f] Agreement between predicted and measured estrogenicity for environmental samples, data in Figure 3. [g] Based on the factors above as well as performance with synthetic samples (SI Table S1). [h] Below average due to its poor method quantification limit. [i] This project in fact intended to run the KBluc assay in two independent laboratories, but one laboratory was not able to develop the KBluc assay within defined QA/QC parameters before the project deadline. [j] Octylphenol creeping across the assay plate appears to be a significant problem in this assay, resulting in variable results when octylphenol was present at high concentrations in the samples.

samples as long as their limitations and technical requirements are clearly understood by the researcher.

Future Work. Further work should involve standardization of operating protocols and quality assurance/quality control procedures, including the development of a standardized bioassay data analysis methodology. It should be emphasized that although this work clearly shows that in vitro bioassays are sufficiently advanced to be used as screening tools for estrogenic contaminants in water, there is still much work to be done to link effects in vitro to whole organism in vivo responses. Recent studies suggests good agreement between in vitro and in vivo estrogenicity (*27, 31*), so careful extrapolation may be possible.

While the bioassays evaluated in this study can measure estrogenic activity in environmental samples, more work is needed to identify trigger levels for further investigation. Indeed, as some of these bioassays can detect estrogenic activity as low as few pg/L of estradiol equivalents, it becomes important to clearly understand and communicate levels of associated risks. In the case of estrogenic activity for example, based on studies with fish (*32–35*), a concentration of 1 ng/L estradiol equivalent may be acceptable. As our analytical detection limits constantly improve, it is crucial to understand that we will be able to detect lower and lower levels of pollution, and that there are thresholds of toxicological concerns below which health risks are not implicated.

Acknowledgments

This project was jointly funded by the Global Water Research Coalition members Water Research Foundation, UK Water Industry Research, Water Environment Research Foundation, CRC for Water Quality and Treatment and Water Services Association of Australia. We thank Natalie Aneck-Hahn, Kathy Bobseine, Chad Blystone, Anne Colville, Viviane Huteau, Benjamin Tan, Katherine Trought, and Catherine van Zijl for assistance in the laboratory and Frans Schulting, Margaret Stewart, Seth Kullman, Deb Lester, Elaine Francis, Djanette Khiari, Gordon Wheale, Issy Cafoor, Peter Donlon, and Maud Achard for editorial comments.

Supporting Information Available

Table S1 contains the EC50 values for 17β-estradiol and the estrogenic equivalency factor of all model compounds used in this study relative to 17β-estradiol. This material is available free of charge via the Internet at http://pubs.acs.org.

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ES903899D

Supplementary information for **Comparison of five in vitro bioassays to measure estrogenic activity in environmental waters** by FDL Leusch, C de Jager, Y Levi, R Lim, L Puijker, F Sacher, LA Tremblay, Vickie S. Wilson, and Heather F. Chapman.

This supplementary information contains Table S1 plus references, a total 4 pages (including this cover sheet).

Table S1. EC50 values for 17β-estradiol (in M) and estrogenic equivalency factor (EEF) of model compounds relative to 17β-estradiol (all values log10).

Chemical	YES	ER-CALUX	MELN	T47D-KBluc	E-SCREEN
17β-Estradiol (E2) [a] CASRN: 50-28-2	-9.60 [1] -9.80 [2] -9.68 [3] -9.40 [4] -9.09 [5] -9.68 [6] -9.64 (*) -9.04 (*)	-11.00 [7] -11.30 [8] -11.22 [9] -11.70 (*)	-10.75 [10] -10.70 [11] -11.25 (*)	-11.52 [12] -11.52 (*)	-11.00 [13] -12.12 [4] -11.05 [14] -10.49 [6] -11.21 [15] -11.31 (*) -11.27 (*)
4-Nonylphenol (4-NP) CASRN: 84852-15-3	-4.66 [16] -3.24 [8] -4.00 [2] -4.60 [3] -6.14 [6] -3.57 (*) -2.96 (*)	-4.64 [8] -4.64 [9] -3.92 (*)	-5.80 [10] -5.02 (*)	-4.43 (*)	-4.89 [17] -4.54 [16] -4.11 [18] -6.63 [4] -4.24 [14] -4.12 [15] -4.41 (*) -4.16 (*)
4-*tert*-Octylphenol (4tOP) CASRN: 140-66-9	-3.32 [5] -5.44 [16] -2.67 (*) -2.77 (*)	cytotoxic (*)	-5.32 (*)	-4.72 (*)	-4.19 [18] -4.01 [14] -4.12 [15] -3.22 (*) -3.38 (*)
Bisphenol A (BA) CASRN: 80-05-7	-5.00 [8] -1.99 [1] -3.96 [3] -4.25 [4] -4.07 [5] -4.30 [16]	-4.48 [7] -5.11 [8] -5.11 [9]	-3.74 [10]	-5.70 (*)	-4.60 [17] -4.53 [18] -5.60 [4] -4.10 [14] -4.28 [15] -4.78 [16] -4.43 (*)
Benzyl butyl phthalate (BBP) CASRN: 85-68-7	-6.00 [8] -5.40 [16] -4.84 (*) ND (-5.90) (*)	-5.85 [8] -5.43 (*)	-5.88 (*)	-5.51 (*)	-6.50 [4] -5.62 [15] -5.60 [16] -5.80 (*) -5.54 (*)
Tetrabromobisphenol A CASRN: 79-94-7	cytotoxic (*) ND (-5.47) (*)	ND [7] -6.88 (*)	-6.50 (*)	-6.37 (*)	-6.00 [19] -5.61 (*) ND (6.87) (*)
Dieldrin CASRN: 60-57-1	NA	-6.62 [9]	NA	NA	-6.70 [13]
Endosulfan CASRN: 115-29-7	NA	-6.00 [9]	NA	NA	-6.00 [13] -6.04 [4] -5.32 [15] -5.84 [16]
p,p'-DDT CASRN: 50-29-3	-3.86 [1] ND (-5.76) (*) ND (-5.60) (*)	-6.02 (*)	-5.87 (*)	-6.17 (*)	-5.40 [16] -6.41 (*) -4.74 (*)
Genistein CASRN: 446-72-0	-3.61 [1] -3.31 [16]	-4.22 [9]	-3.19 [10]	-4.52 [12]	-4.89 [17] -3.55 [15] -3.85 [16] -4.05 (*)
Estrone (E1) CASRN: 53-16-7	-1.00 [8] -0.40 [2] -0.42 [20] -1.02 [16] -0.61 (*)	-1.25 [8]	-1.60 [10] -0.60 [11]	-1.67 (*)	-2.00 [17] -1.91 [18] -0.87 [14] -1.02 [15] -1.36 [16] -1.95 (*)
Estriol (E3) CASRN: 50-27-1	-2.62 [20] -2.20 [16]	0.00 [21]	-0.75 [10] -1.09 [11]	-1.32 (*)	-1.15 [17] -0.53 [14] -0.60 [16] -1.07 (*)
Ethynylestradiol (EE2) CASRN: 57-63-6	0.08 [8] -0.05 [2] 0.08 [3] 0.36 [4] -0.02 [5] -0.15 [6] -0.05 [16] 0.09 (*)	0.08 [8]	0.39 [10] 0.06 [11]	-0.45 [12]	0.10 [17] 0.03 [8] -0.78 [4] 0.13 [14] 0.28 [6] -0.04 [15] 0.05 [16] -0.17 (*)
Tamoxifen CASRN: 10540-29-1	-3.15 [4] -4.33 [16] -3.07 (*) -3.40 (*)	-3.83 (*)	-3.92 (*)	-5.52 [12] -3.08 (*)	-4.39 [17] -3.30 [16] -3.25 (*) -3.22 (*)

CASRN: Chemical Abstract Services Registry Number. ND = no detectable effect (although when available, the highest possible potency is given in brackets based on the highest concentration tested). NA = not available. Note that Fang et al 2000 [16] is a review and reports potencies from several different papers. [a] Note that for 17β-estradiol, the log of the EC50 (in M) is reported instead of the log of the relative potency (the log of the relative potency of 17β-estradiol is of course 0.00). (*) Value obtained in this study by dividing the EC50 of 17β-estradiol by the EC50 of the compound.

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