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ORIGINAL ARTICLE

Comparison of two yeast bioluminescent assays applied to water monitoring of estrogenic activity

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ABSTRACT

The aim of our study was to compare the performance of two bioluminescent yeast estrogen bioassays, *Saccharomyces cerevisiae* BLYES (*lux*-based) and *S. cerevisiae* BMAEREluc/ERα (*luc*-based), to determine estrogenic activity in environmental samples. Two bioassays and chemical analysis were used to analyze 36 water samples. Samples were solid phase extracted and chemical analysis and two yeast bioassays were used for analysis. Individual estrogenic compounds were also analyzed by each estrogen assay. *S. cerevisiae* BLYES produced positive responses to 15 out of 36 water samples, versus 3 out of 36 samples with BMAEREluc/ERα. Both strains responded to individual chemicals and were able to detect estrogenic substances in environmental samples. However, *S. cerevisiae* BLYES was easier to use, faster, less expensive; requiring fewer handling steps and no exogenous substrate addition. In addition, it appears more sensitive to estrogens in the environmental samples. Because previous studies have found contamination with estrogenic substances worldwide, including some in Brazilian waterways, a method to quickly and inexpensively screen environmental samples for endocrine-active substances in monitoring programs is critically needed. *S. cerevisiae* BLYES was demonstrated to be capable of gauging the estrogenic burden of water samples in a high throughput and sensitive fashion.

Keywords: BLYES, bioluminescent yeast estrogen screen, estrogenicity, water contamination

1. Introduction

Concern over exposure to chemicals from drinking water has increased greatly over the last two decades, even after amendment of the EPA's Safe Drinking Water Act (1996). Current concerns focus on chemicals that may interfere with the human endocrine system (termed endocrine disrupting chemicals or EDCs). Many types of chemicals, including natural and synthetic steroids, pesticides, and plasticizers, are known to interact with the human estrogen receptor (1) and have been linked to a number of undesirable health effects in both humans and wildlife (2-6). In fact, organic contaminants with endocrine activity have been found in waterways in both the U.S. and Brazil (7-11).

Recently, an integrated approach was used to evaluate Brazilian drinking and surface waters for estrogenic burden. Jardim *et al.* (11) combined chemical analysis with bioassays to determine whether drinking waters in the state of São Paulo were contaminated with chemicals that mimic human hormones. They determined that rivers classified as poor (according to the Public Water Supply Quality Index (IAP) published by the State Environmental Agency of São Paulo, CETESB) have concentrations of estrogenic substances measureable with both chemical analysis and bioassays. However, the amount of potential estrogens detected with the bioassays was not fully explained by the amount of each substance detected by chemical analysis, highlighting the benefit of using bioassays to determine the amount of biologically available EDCs present in water samples. In addition, Bergamasco *et al.* (10) demonstrated the utility of these assays for similar samples from the state of São Paulo and highlighted the possible use of these assays in monitoring programs.

Bioassays have been developed to predict the effect of these chemicals on vertebrates because humans and wildlife are exposed to increasing numbers and amounts of chemicals. The scope of testing needed is so large that EPA has estimated that more than 87,000 new and existing chemicals need to be screened (12). Therefore bioassays are a good first tier screening method to quickly determine if samples contain chemicals with the potential to interfere with human endocrine functioning. These assays must be maximally suitable for high throughput use,

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inexpensive, quick, easy to use, and most importantly sensitive to detect chemicals found in low concentrations (7, 9).

Several bioassays, in mammalian cells and in yeast, have been developed to detect a chemical's potential for endocrine disruption. Some use a colorimetric-based system in which the yeast, *Saccharomyces cerevisiae,* harboring the human estrogen receptor on its genome was transformed with plasmids that break down a chromogenic substrate in the presence of a potential estrogen (13). While these types of assays have been used extensively to study chemicals like PCBs e.g. Layton *et al.* (14), they require the addition of the substrate CPRG, which is quite costly and cumbersome. In addition, it takes 3-5 days to detect a response from these strains, limiting their use in high throughput systems. The next-generation of estrogen-sensing bioassays was based on genetically engineering yeast to produce a bioluminescent signal rather than a colorimetric one. Some bioassays use *luc* genes from the firefly *Photinus pyralis* e.g. strain *S. cerevisiae* BMAEREluc/ERα (15). This strain, as with other *luc*-based assays, requires the addition of a substrate, which adds both cost and labor, but these types of assays have been purported to be more sensitive than *lux*-based assays.

Lux-based bioassays, like *S. cerevisiae* BLYES (1) generate bioluminescence using *Photorhabdus luminescens lux* genes and may contain the full *lux* operon (genes *luxCDABE*) thereby producing the substrate as well as the enzyme for the reaction. This creates a fully autonomous bioreporter under control of estrogen response elements, with the benefit of both decreased cost and increased ease of use. To be optimally useful as high throughput environmental or chemical detection systems for estrogens, the bioassays must also be rapid and sensitive. The two most commonly used yeast-based, estrogen-sensing bioassays are *S. cerevisiae* BMAEREluc/ERα (15) and *S. cerevisiae* BLYES (16). Both bioassay strains contain a constitutively produced human estrogen receptor α gene on the genome and bioluminescence genes on a plasmid, under control of estrogen response elements (*luc* genes in BMAEREluc/ERα and *lux* genes in BLYES). In an effort to compare how these two prominent yeast-based bioassays function in terms of speed, sensitivity, and ease of use, we exposed the strains of Leskinen *et al.* (15) and of Sanseverino *et al.* (1) to a series of individual chemicals and environmental samples, as well as performed chemical analysis on the same samples (10).

2. Materials and methods

2.1. Sampling

Water samples were collected in surface water bodies and taps in the State of São Paulo, Brazil. Briefly, raw surface water samples (1 L) were collected in rivers or reservoirs used for drinking water production in the cities Guarulhos (Tanque River Reservoir), Campinas (Atibaia River), Atibaia (Atibaia River), Cerquilho (Sorocaba River), and Barueri (Cotia River). Surface water samples were collected as close as possible to the source of drinking water intakes. These waters were classified as having excellent, good, intermediate, fair, and poor water quality, respectively, according to the Public Water Supply Quality Index (IAP). This classification was developed by the São Paulo State Environmental Agency (CETESB) and is based on a combination of pathogen and physical/chemical analyses. Corresponding tap water samples (4 L) were collected from the municipal distribution system. In addition, a raw sewage grab sample was collected from the wastewater effluent from a hospital and was expected to contain large concentrations of estrogenic substances. Samples were collected between April 2009 and March 2010. In this period the samples were collected during a short dry period. Water was collected in clean amber glass bottles and transported to the laboratory on ice. The samples were extracted within 24 hours.

2.2. Solid phase extraction

Samples were solid phase extracted according to Sodré *et al.* (17), the extracts divided and used for both bioassays and chemical analysis. Briefly, samples were filtered through 0.45 µm pore size cellulose acetate membranes (Sartorius, Gottingen, Germany) with solid phase extraction performed using 500 mg HLB Oasis cartridges (Waters, Milford, USA) fitted to a custom extraction system (18). One liter of raw water and 4 L of drinking water were extracted using this method. This method consists of conditioning the cartridges with methanol (6 mL) and then passing filtered samples through the cartridge at a flow rate of 9 ± 1 mL min^{-1}. Elution was performed using a total of 6 mL methanol in a 12-port Prep SPE vacuum manifold (Fisher Scientific, Fair Lawn, USA). Eluates were collected in glass vials and evaporated under ultra-pure nitrogen gas. Samples were split into three aliquots, with one for each bioassay and one for chemical analysis.

2.3. Chemical Analysis

Five endocrine disrupting compounds were measured in the samples: estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethinylestradiol (EE2), and diethylstilbestrol (DES), which were acquired from Sigma–Aldrich (St. Louis, USA) (Table 1). These compounds were quantified by liquid chromatography tandem mass spectrometry (Agilent 1200 LC system coupled to an Agilent 6410 TripleQuad mass spectrometer). The chromatographic separation (Zorbax SB-C18, 2.1, 30 mm, 3.5 um, Agilent Technologies) was achieved with gradient elution using ammonium hydroxide solutions (0.1% v/v) in reagent water and in methanol as the mobile phase. The triple quadrupole mass spectrometer was operated in the electrospray negative ionization mode. Data acquisition was performed by multiple reactions monitoring (MRM), recording the transitions between the precursor ion and at three product ions for each target analyte. Details of the LC-MS/MS parameters are presented in Table 2. The analytical method exhibits good linearity for all compounds in the range of 0.5–10 ng L^{-1}. Determination coefficients (R^2 values) for linear equations were higher than 0.884. Limit of detection for the five compounds varied between 0.3 and 4.2 ng L^{-1}. Average recovery for a chemical spiked into surface water at 10 ng L^{-1} varied between 58% and 79% and in tap water varied between 42% and 83%.

Table 1. Chemicals used in this study.

Chemical	Formula	Molecular Weight (g mol^{-1})	Abbreviation	CAS[a] Number	Merchant	Purity (%)
Estrone	$C_{18}H_{22}O_2$	270.16	E1	53-16-7	Aldrich	99 +
17β-estradiol	$C_{18}H_{24}O_2$	272.18	E2	50-28-2	Aldrich	97
Estriol	$C_{18}H_{20}O_3$	288.17	E3	50-27-1	Fluka	97 +
17α-ethinylestradiol	$C_{20}H_{24}O_2$	296.18	EE2	57-63-6	Aldrich	98
Diethylstilbestrol	$C_{18}H_{20}O_2$	268.12	DES	56-53-1	Sigma	99 +

[a]CAS: Chemical Abstracts Service.

Table 2: LC-MS/MS conditions used to quantify six endocrine disrupting compounds in natural waters.

Compounds	Polarity	Fragmentor (V)	Precursor ion (m/z)	Product ions / Collision (V)			Limit of Detection
				m/z (1)	m/z (2)	m/z (3)	(ng L^{-1})
E1	-	100	269.1	144.9 / 30	143.0/ 40	182.9/ 35	0.8
E2	-	90	271.1	183.0/ 35	143.0/ 40	144.9/ 30	1.8
E3	-	110	287.1	170.9/ 30	143.0/ 40	144.9/ 35	0.3
EE2	-	100	295.1	144.9/ 30	143.0/ 40	158.9/ 30	4.2
DES	-	130	267.2	220.0/ 30	237.1/ 25	251.1/ 15	1.0

*m/z ± 0.1

2.4. BLYES Bioassay

S. cerevisiae BLYES and BLYR strains were employed according to Sanseverino *et al.* (16, 1). Briefly, cells were grown to an OD_{600} 1.0 overnight at 30ºC in yeast minimal medium as described in Routedge and Sumpter (13). Extracts were serially diluted nine times generating a four-fold range of concentrations, and then 100 µl plated into 96-well plates. All dilutions were performed in 2% DMSO (Sigma-Aldrich, St. Louis, USA). The yeast strains (100 µl) were added to the samples and incubated at 30ºC for approximately three hours. The final extract concentration of DMSO was 1% in samples and controls; the highest exposure concentration was 100x. Bioluminescence was read using a Victor X3 Multilabel Plate Reader (PerkinElmer, Waltham, USA). Negative controls consisted of wells with a) DMSO and water and b) only ultrapure water. Results were graphed and analyzed according to Sanseverino *et al.* (1), except that bioluminescence values were graphed as a percent of the maximum bioluminescence response. Bioluminescence values were graphed versus concentration of the sample (Figures 1-3). A standard curve was generated by spotting a set of 18 dilutions of 17β-estradiol (E2) on each plate (concentration range of $1x10^{-7}$ M to $2.5x10^{-13}$ M). Data from positive samples were used to calculate the concentration at which the assay is 50% active (EC_{50}) by linear regression. A regression was performed in SigmaPlot (Systat, San Jose, CA) on the values along the linear portion of the dose-response curve and then the concentration that represents the midpoint of the linear portion of the curve was determined, as detailed in Sanseverino *et al.* (16, 1). For environmental samples, the EC_{50} of E2 from that assay run was divided by the EC_{50} of the sample to determine the molar concentration of "estrogenic equivalents" present in samples and is reported as ng E2 equivalents per liter. For individual chemicals, estrogenic potency (TEQ) was calculated as the ratio of the EC_{50} of E2 from that assay run to EC_{50} of the chemical.

2.5. BMAERELuc/ERα Bioassay

S. cerevisiae BMAERELuc/ERα assay was performed according to Leskinen *et al.* (15). Cultures were grown overnight at 30ºC in yeast synthetic drop out media (15), then diluted to OD_{600} 0.4 and grown to OD_{600} 0.6. Solid phase extracts were serially diluted in 10% DMSO and pipetted (10 µL) into 96-well plates, along with 90 µL of yeast strains. The final concentration of DMSO was 1% in samples and controls; the highest concentration that this strain was exposed to was 100x. A standard curve was generated by spotting a set of 10 dilutions of 17β-estradiol (E2) on each plate (concentration range of $1x10^{-7}$ M to $2.5x10^{-11}$ M). The microplates were then incubated at 30ºC for 2.5 h. One hundred µL of 1 mM D-luciferin (in 0.1 M Na-citrate buffer) were added to each well and bioluminescence was read 5 minutes later using the Victor X3 Multilabel Plate Reader (PerkinElmer, Waltham, USA). Negative controls consisted of wells with a) DMSO and water and b) only ultrapure water.

Responses higher than three times the standard deviation plus the mean of the blanks were considered positive. Estrogenic potency and EC_{50} were determined for individual chemicals as described above.

3. Results

3.1. Analysis of estrogenic substances

Pure chemicals were tested with both bioassays and EC_{50}, detection limit, and relative potency in relation to E2 of each chemical was calculated. The detection limits in reference to E2 were 6.9 ng L^{-1} for BLYES and 73 ng L^{-1} for BMAEREluc/ERα but given that the yeast were exposed to environmental samples that were 100x concentrated the actual detection limit for natural samples was much lower. The EC_{50} is the concentration of chemical at which the assay is half-maximally active and is used routinely to compare different types of assays. In our study, the EC_{50} for E2 was 46.3 ng L^{-1} (1.7 x 10^{-10} mol L^{-1}) with BLYES and 1,470 ng L^{-1} (5.4 x 10^{-9} mol L^{-1}) with BMAEREluc/ERα (Figure 1).

Relative potency is used to assess how much more or less potent a chemical is than the reference chemical E2. It is calculated in reference to E2 by computing the ratio of the EC_{50} of E2 to the EC_{50} of the chemical. Chemicals that cause a greater estrogenic response will have a lower EC_{50} and a higher relative potency. Figures 2 and 3 display the response to individual chemicals obtained for the BMAEREluc/ERα and BLYES, respectively. The synthetic estrogen EE2 caused a higher response than the natural estrogen E2 with both BLYES and BMAEREluc/ERα (Table 3). BMAEREluc/ERα produced a 5x greater response to EE2 than E2 while BLYES responded 2.7x more strongly.

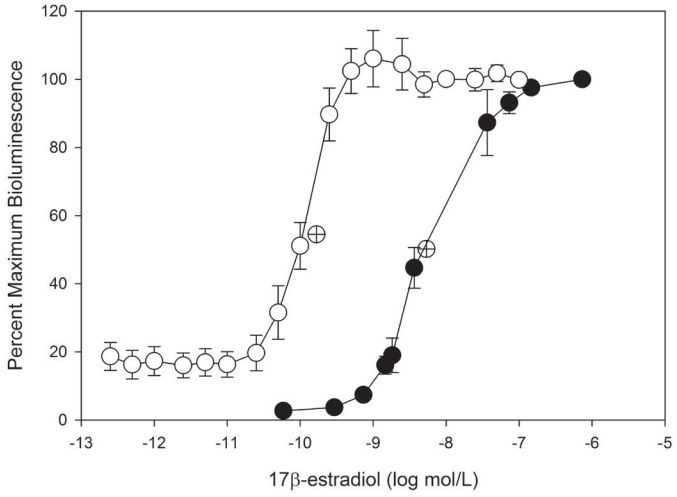

Figure 1. Comparison of BMAEREluc/ERα and BLYES response to 17β-estradiol (E2). The response to E2 for BMAEREluc/ERα (●) and BLYES (○) yields an EC_{50} of 5.4 x 10^{-9} mol L^{-1} (1470 ng L^{-1}) and 1.7 x 10^{-10} mol L^{-1} (46.3 ng L^{-1}), respectively (indicated by crossed circles on each corresponding graph). Data represents the mean and standard deviation of four independent assays for BMAEREluc/ERα and five independent assays for BLYES.

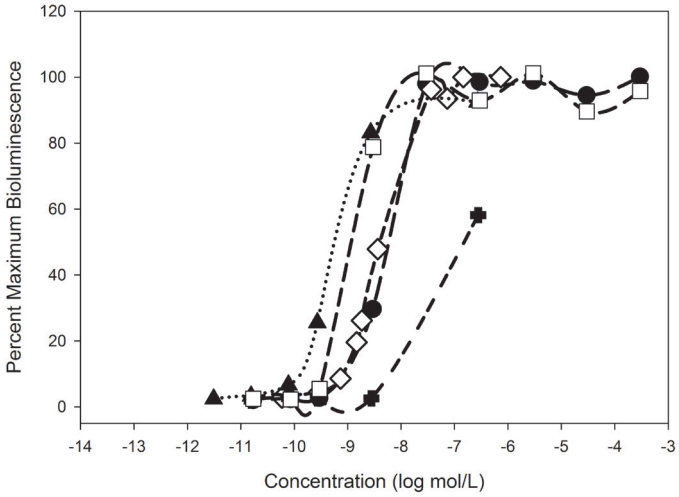

Figure 2. Response curves of *Saccharomyces cerevisiae* BMAEREluc/ERα to pure chemicals E1 (●), E2 (◊), EE2 (▲), E3 (+), and DES (□). Results are expressed in terms of percentage of maximum response versus log of the molar concentration of the chemicals.

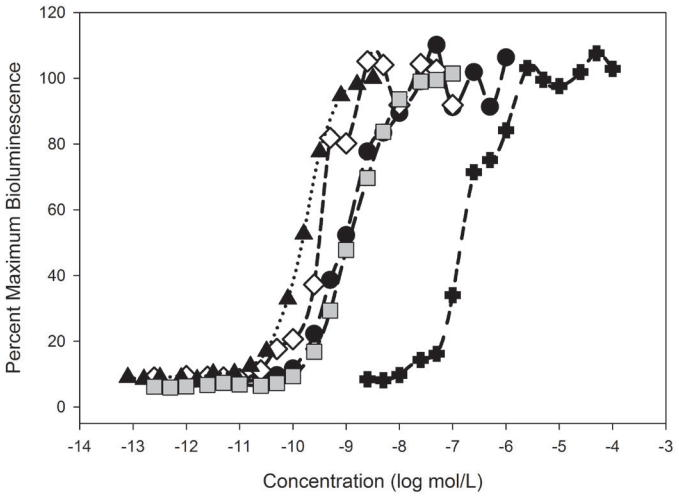

Figure 3: Response curves of *Saccharomyces cerevisiae* BLYES to pure chemicals E1 (●), E2 (◊), EE2 (▲), E3 (+), and DES (□). Results are expressed in terms of percentage of maximum response versus log of the molar concentration of the chemicals.

The detected relative potency of the compounds followed the pattern of (strongest to weakest response) EE2 > E2 > E1 > E3 for both bioassays, with the only difference being the actual calculated value of relative potency. With the natural estrogen estrone (E1), BLYES demonstrated a 13x lower response than for E2 and BMAEREluc/ERα produced a 2x lower response (Table 3). The natural estrogen estriol (E3) produced the biggest difference in relative potency between the two assays; BLYES demonstrated 1,639x lower response than E2 whereas BMAEREluc/ERα produced 19x less potency but the two strains had similar EC_{50}s for this chemical. For the synthetic estrogen

Table 3: Estrogenic activity and relative potency (TEQ) obtained in this study and as reported in Bergamasco *et al.* (2011).

Chemical	Strain / Method	Estrogenic Activity (EC_{50}) µg L^{-1}	Relative Potency (TEQ)
Estrone (E1)	BLYES[a]	0.59	7.8×10^{-2}
	BMAEREluc/ERα	2.6	0.55
17β-Estradiol (E2)	BLYES	0.046 (± 0.0093)	1
	BMAEREluc/ERα	1.5 (± 0.68)	1
Estriol (E3)	BLYES[a]	75	6.1×10^{-4}
	BMAEREluc/ERα	29	5.2×10^{-2}
17α-Ethinylestradiol (EE2)	BLYES[a]	0.017	2.7
	BMAEREluc/ERα	0.30	5.0
Diethylstilbestrol (DES)	BLYES	0.78	5.9×10^{-2}
	BMAEREluc/ERα	0.56	2.7

EC_{50}: half-maximal activity for the assay.
TEQ: potency relative to E2 (calculated as EC_{50} of E2 divided by EC_{50} of the chemical).
[a]Data reported in Bergamasco *et al.* (2011), presented for comparison to strain BMAEREluc/ERα.

diethylstilbestrol (DES), the EC_{50} for BLYES and BMAEREluc/ERα are nearly identical but because the EC_{50} for E2 is different with strain BMAEREluc/ERα, the relative potencies in relation to E2 are also different. BLYES demonstrated 17x less response than E2 whereas BMAEREluc/ERα produced 3x greater response to DES. In all cases, the maximum response observed with the pure chemicals matched that of the maximum response with E2, indicating that each of these chemicals was able to produce a full dose-response in the bioassays. Results for BLYES were similar to Sanseverino *et al.* (16, 1) who have measured EC_{50} and relative potency for numerous chemicals of many different classes (e.g. pharmaceuticals, hormones, and pesticides), whereas the results for BMAEREluc/ERα indicated that the strain was less sensitive than previously reported. Despite the differences in sensitivity, EE2 was the most potent, followed by E2, E1, and then E3.

3.2. Analysis of water samples

Surface and tap water samples were collected from five locations in Brazil at three times for raw surface water and four times for tap water over the course of one year, beginning in April 2009 (Table 4). The samples were collected from locations with different degrees of contamination as detailed in methods. In this study, 15 raw surface water samples were tested and estrogenic activity was detected in the majority these samples (12 out of 15 with strain BLYES). Interestingly, in the 20 treated (tap) water samples, 19 produced no estrogenic substances as measured by both bioassays and chemical analysis. BLYES produced similar responses to the chemical analysis; in each case when chemical analysis detected an estrogenic substance the BLYES strain also detected a response. In addition, in the sample that the chemical analysis detected its highest response (the hospital effluent sample), BLYES also produced its highest response (Table 4).

In samples where both strains detected a response, the responses were in the same order of magnitude e.g. Barueri surface water samples collected in June 2009 presented 3.1 and 1.9 ng E2 equivalents L^{-1} as determined by strain BLYES and BMAEREluc/ERα, respectively. However, the BLYES strain detected responses in more samples than did strain BMAEREluc/ERα (15 out of 36 as opposed to 3 out of 36), indicating that the BLYES was more sensitive for environmental samples. For example, the Campinas surface water sample from June 2009 presented estrogenic activity of 0.13 ng E2 equivalents L^{-1} with BLYES and 1.48 ng L^{-1} of E3 were detected with chemical analysis but no response was observed with BMAEREluc/ERα.

4. Discussion

In all cases where chemical analysis was able to detect an estrogenic substance, the amount of that substance failed to fully explain the observed response with the bioassays. This indicates several possible scenarios: a) another compound was present with the potential to activate the estrogen-sensing system of the BLYES bioassay, b) strong estrogens were present below the chemical detection limit but above the bioassay detection limit, c) small amounts of multiple estrogens were able to produce an additive response high enough to be detected with bioassays, or d) synergistic interactions were taking place in these samples. The source of this potential estrogenic activity is unknown but this highlights the point that chemical analysis alone cannot possibly analyze all estrogenic substances present in complex samples. Therefore, bioassays may be more useful for screening large numbers of samples with unknown chemical content in that they are able to quickly screen for bioavailable estrogens. This would be particularly important in monitoring programs. Sites presenting positive responses in the bioassay could be

Table 4. Results of treated and raw surface water sample analysis with both bioassays and chemical detection. Samples were collected in April, June, and September 2009 and March 2010. Waterways are classified as excellent (Guarulhos), good (Campinas), intermediate (Atibaia), fair (Cerquilho), and poor (Barueri) according to the Public Water Supply Quality Index (IAP). Results are presented in terms of ng E2 equivalents L^{-1} for bioassay data and in terms of ng L^{-1} for chemical analysis data. BLYES and chemical raw water data has previously been published in Bergamasco *et al.* (2011) and is presented for comparison to the BMAEREluc/ERα strain.

Sample (IAP water quality)	Date collected	Chemical detected by chromatographic analysis[a]	Calculated estrogenic equivalents[b] (ng E2 equiv. L^{-1})	BLYES (ng E2 equiv. L^{-1})	BMAEREluc/ ERα (ng E2 equiv. L^{-1})
Guarulhos-treated water (excellent)	April-09	BDL[c]	BDL	BDL	BDL
	June-09	BDL	BDL	BDL	BDL
	Sept-09	BDL	BDL	BDL	BDL
	March-10	BDL	BDL	BDL	BDL
Guarulhos- raw surface water (excellent)	June-09	BDL	BDL	BDL	BDL
	Sept-09	BDL	BDL	0.21	BDL
	March-10	BDL	BDL	0.06	BDL
Campinas-treated water (good)	April-09	BDL	BDL	BDL	BDL
	June-09	BDL	BDL	BDL	BDL
	Sept-09	BDL	BDL	BDL	BDL
	March-10	BDL	BDL	BDL	BDL
Campinas- raw surface water (good)	June-09	E3 1.5 ng L^{-1}	9.8×10^{-4}	0.13	BDL
	Sept-09	BDL	BDL	0.33	BDL
	March-10	BDL	BDL	0.22	BDL
Atibaia-treated water (intermediate)	April-09	BDL	BDL	BDL	BDL
	June-09	BDL	BDL	BDL	BDL
	Sept-09	BDL	BDL	BDL	BDL
	March-10	BDL	BDL	BDL	BDL
Atibaia- raw surface water (intermediate)	June-09	BDL	BDL	BDL	BDL
	Sept-09	E1 0.8 ng L^{-1}	6.4×10^{-2}	0.33	BDL
	March-10	BDL	BDL	0.42	BDL
Cerquilho-treated water (fair)	April-09	BDL	BDL	BDL	BDL
	June-09	BDL	BDL	BDL	BDL
	Sept-09	BDL	BDL	BDL	BDL
	March-10	BDL	BDL	BDL	BDL
Cerquilho- raw surface water (fair)	June-09	BDL	BDL	0.70	BDL
	Sept-09	E1 0.8 ng L^{-1}	6.4×10^{-2}	2.1	9.0
	March-10	E1 2.6 ng L^{-1}	0.21	0.53	BDL
Barueri-treated water (poor)	April-09	BDL	BDL	0.23	BDL
	June-09	BDL	BDL	BDL	BDL
	Sept-09	BDL	BDL	BDL	BDL
	March-10	BDL	BDL	BDL	BDL
Barueri- raw surface water (poor)	June-09	E3 7.7 ng L^{-1}	5.0×10^{-3}	3.1	1.9
	Sept-09	E3 3.8 ng L^{-1}	2.5×10^{-3}	7.1	BDL
	March-10	E3 18.3 ng L^{-1}	1.2×10^{-2}	0.35	BDL
Hospital Effluent	June-09	E2 5.6 ng L^{-1} E3 182 ng L^{-1}	5.7	20.5	13.1
Blank		BDL	BDL	BDL	BDL

[a]Chromatographic analysis by LC/MS-MS performed by the Environmental Chemistry Lab at UNICAMP.
[b]Estrogenic equivalents: calculated by multiplying the chemical analysis value by the relative potency of the strain BLYES.
[c]BDL: below detection limit.

prioritized and selected for further analyses, including chemical identification of the estrogenic compounds for appropriate corrective and/or mitigation actions.

The strain *S. cerevisiae* BLYES was easier to handle, not requiring multiple phases of dilution and growth or addition of substrate. In addition, it was more sensitive for the detection of estrogenicity in environmental samples than *S. cerevisiae* BMAEREluc/ERα. The reported EC_{50} of E2 for the two assays is similar; 65 ng L^{-1} (2.4 ± 1.0 x 10^{-10} mol L^{-1}) with BLYES (16) and 136 ng L^{-1} (5 x 10^{-10} mol L^{-1}) for BMAEREluc/ERα (15). In this study we observed an EC_{50} and detection limit for BMAEREluc/ERα higher than published data, indicating a lower sensitivity with this isolate than previously reported. Sensitivity of genetically engineered strains can vary within laboratories and over time. Bioassays were performed according to the established protocol for each strain and it is possible that if methods were standardized across both strains (e.g. using the same amount of cells in each assay) they may perform with equal sensitivity to environmental samples.

The greater response of BLYES to EE2 (18x greater response than BMAEREluc/ERα) was interesting and may be one possible reason why BLYES responded more strongly to environmental samples, since EE2 is an estrogen found in birth control pills and would be expected to be present in water samples, albeit at low concentrations. Concentrations of EE2 below the chemical detection limit were still able to cause a positive response with the bioassay; therefore the fact that we did not detect EE2 with chemical analysis does not preclude its presence in samples. The chemical detection limit of EE2 was 4.2 ng L^{-1}, while for natural samples the detection limit of the BLYES bioassay is 0.07 ng E2 equivalents per liter. If a sample contains 3 ng L^{-1} EE2, it would not be detectable with chemical analysis but it would be detectable with the BLYES bioassay (as 9 ng E2 equivalents L^{-1} given the relative potency of EE2).

Jardim *et al.* (11) have detected estrogenic substances in Brazilian waters using an integrated approach, with both chemical analysis and bioassays. They demonstrated that the highest concentration of estrogens was in the river with poor classification (Cotia River in Barueri), which is known to receive both raw and treated sewage from a neighboring urban area. This result is significant because this water is used to produce drinking water for the city of Barueri and represents a potential source of contamination to the inhabitants. Interestingly, one positive response was seen in a drinking water sample from the tap water in Barueri in April 2009 (Table 4) but more samples would need to be tested to confirm this result.

Given that Brazilian surface waters are contaminated with EDCs, it would be helpful to have screening methods that are sensitive, easy to handle, and inexpensive. Even in surface waters classified as having good water quality, estrogenic substances were observed (10, 11). The waters from Campinas region, although they have a good classification, produced estrogenic responses in every raw surface water sample tested. Because large numbers of environmental samples will need to be screened, bioassays capable of handling large numbers of samples should be maximally high throughput. BLYES is easier to use and less expensive, since it produces its own substrate for the bioluminescence reaction. Strains BMAEREluc/ERα and YES both require the addition of a substrate for the reaction, which adds both cost and additional handling to the assays. Because strain BLYES was demonstrated to be the easiest and cheapest assay, and given that in this study it was more sensitive for environmental samples, it is a good choice for monitoring studies in which large numbers of samples are processed.

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