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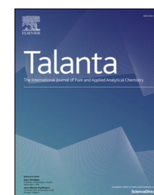

Microcarrier-based fluorescent yeast estrogen screen assay for fast determination of endocrine disrupting compounds

Bruno J.R. Gregório [a], Inês I. Ramos [a], Sara S. Marques [a], Luísa Barreiros [a,b], Luís M. Magalhães [c], Rudolf J. Schneider [d], Marcela A. Segundo [a,*]

[a] LAQV, REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313, Porto, Portugal
[b] Escola Superior de Saúde, Instituto Politécnico do Porto, Rua Dr. António Bernardino de Almeida 400, 4200-072, Porto, Portugal
[c] Present affiliation: Research & Development, BIAL- Portela & C[a], S.A., Coronado (S. Mamede e S. Romão), Portugal
[d] Department of Analytical Chemistry, Reference Materials, BAM Federal Institute for Materials Research and Testing, Richard-Willstätter-Strasse 11, D-12489, Berlin, Germany

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ABSTRACT

The presence of endocrine-disrupting compounds (EDCs) in water poses a significant threat to human and animal health, as recognized by regulatory agencies throughout the world. The Yeast Estrogen Screen (YES) assay is an excellent method to evaluate the presence of these compounds in water due to its simplicity and capacity to assess the bioaccessible forms/fractions of these compounds. In the presence of a compound with estrogenic activity, *Saccharomyces cerevisiae* cells, containing a lacZ reporter gene encoding the enzyme β-galactosidase, are induced, the enzyme is synthesised, and released to the extracellular medium. In this work, a YES-based approach encompassing the use of a lacZ reporter gene modified strain of *S. cerevisiae*, microcarriers as solid support, and a fluorescent substrate, fluorescein di-β-D-galactopyranoside, is proposed, allowing for the assessment of EDCs' presence after only 2 h of incubation. The proposed method provided an EC50 of 0.17 ± 0.03 nM and an LLOQ of 0.03 nM, expressed as 17β-estradiol. The assessment of different EDCs provided EC50 values between 0.16 and 1.2×10^3 nM. After application to wastewaters, similar results were obtained for EDCs screening, much faster, compared to the conventional 45 h spectrophotometric procedure using a commercial kit, showing potential for onsite high-throughput screening of environmental contamination.

1. Introduction

An endocrine-disrupting compound (EDC) is, as defined by the World Health Organization, "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" [1]. EDCs are recognized as a threat to public health, being associated with perinatal, developmental, metabolic, and reproductive detrimental effects, such as obesity, intelligence quotient (IQ) loss and intellectual disability, among others [2]. It is estimated that exposure to EDCs cost was more than 340 billion dollars in the USA and more than 200 billion dollars in the EU in 2010 [3–5]. These compounds can be found in plastics (plasticizers), personal care products, pesticides [3,6], and in many pharmaceutical formulations directed to the endocrine system [6]. Even though most of them are degraded in wastewater treatment plants, not all xenobiotics are removed from the effluent and may reach water courses [7].

The European Parliament and the Council of the European Union enacted a Water Framework Directive (2000/60/EC), where the action framework in the field of water was established and the importance of the monitoring of water was stressed [8]. Later on, the directive 2008/105/EC has established a watchlist of substances to be monitored, where some EDCs were included [9] and, in 2015, compounds such as 17α-ethynylestradiol (EE2), 17β-estradiol (E2) and estrone (E1) were introduced in the watchlist. Even though the monitoring obligation has ceased in 2019, monitoring data will still be considered in the risk assessment of each substance, and will be reintroduced in the next watchlist [8,10]. Other compounds with endocrine disrupting properties, like octocrylene or benzophenone-3, were introduced in the most recent watchlist [11]. Furthermore, E2 and nonylphenol were introduced in the watchlist for substances and compounds of concern for water intended for human consumption [10]. This concern of the

* Corresponding author.
E-mail address: msegundo@ff.up.pt (M.A. Segundo).

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European Union regulatory bodies shows that the determination of EDCs in water is of uttermost importance.

There are several approaches that can be implemented for the evaluation of EDCs in water, such as LC/MS-MS, that is routinely employed in laboratories, however it requires expensive equipment and specialized operators [12]. A different approach consists in the use of whole cell-based assays, that are widely employed in the study of contamination and in toxicological assays, allowing for the assessment of the bioaccessible forms/fractions of the compounds of interest, and can be based on yeast or mammalian cells [13,14]. Among these, recombinant yeast assays, and specifically assays based on *Saccharomyces cerevisiae*, are excellent tools as this yeast is simple and inexpensive to handle, does not demand strict growth conditions, and has its genome fully sequenced [15,16]. The available synthetic biology and metabolic engineering tools, like the CRISPR/Cas9 system, permit the modification of the yeast genome and the development of biosensors for analytes of interest [17].

In this work, a strain of *S. cerevisiae* that contains the human estrogen receptor (hER) and a reporter plasmid carrying the reporter gene lacZ that encodes the enzyme β-galactosidase and is controlled by an estrogen-responsive sequence (ERE), is employed. When estrogens are present, they bind to the hER and this complex interacts with the ERE, activating the lacZ reporter gene, culminating in the synthesis of β-galactosidase, that is then released to the extracellular medium [18] (Fig. S1). There, in the presence of a suitable substrate, like the colorimetric chlorophenol red-β-D-galactopyranoside (CPRG) [18,19], or the fluorimetric fluorescein di-β-D-galactopyranoside, the glycosidic bond of the substrate is cleaved, and the released probe is measured by spectrophotometry or fluorimetry, respectively.

Even though fluorescence-based substrates have been employed in the study of β-galactosidase of mammalian, bacterial, or yeast cells, few works have employed them in the YES assay, featuring in this case fluorescein di-β-D-galactopyranoside, resorufin-β-D-galactopyranoside, and 4-methylumbelliferyl-β-D-galactopyranoside [20–22]. Other approaches for the determination of EDCs proposed the integration in the yeast of a plasmid carrying a luciferase gene for bioluminescence detection [14,23,24], or a citrine gene for fluorescence measurements [12], among others.

The use *S. cerevisiae* carrying the lacZ reporter gene encoding β-galactosidase represents a good alternative for EDCs' monitoring, however it requires a few days of incubation, which impair the wider applicability of the assay. The initial protocol required 72 h of incubation [18], while more recent improvements got the time down to 4 h [25]. Alternative approaches using the lacZ reporter gene, such as the planar-YES, have been developed presenting incubation times between 3 and 4 h.

While the traditional YES approach allows for the evaluation of additive, synergistic, and antagonistic biological effects, the planar-YES, thanks to the separative chromatographic run, allows the isolation of compounds with different retention factors, and a possible separation between cytotoxic and estrogenic compounds present in the samples. However, this approach requires the use of organic solvents for the chromatographic separation and a larger volume of yeast cells for dipping the test plates [21,26]. Different works using modified *S. cerevisiae* strains were able to reduce the incubation times to values between 30 min and 1 h, but they have employed other reporter systems, such as green fluorescent protein-based [27] or bioluminescence-based systems [14,24,28]; nevertheless, they are tailored strains that are not commercially available.

Therefore, the main goal of this work was to develop a faster assay for the assessment of the presence of EDCs resorting to lacZ modified *S. cerevisiae* employing a fluorescent enzymatic substrate, fluorescein di-β-D-galactopyranoside, that circumvents the time constraints, bridging the gap between different approaches and reporter systems, while keeping the sensitivity on pair with other yeast cell-based bioassays.

2. Materials and methods

2.1. Chemicals and solutions

All chemicals utilized were of analytical reagent grade with no further purification. Ultrapure water (resistivity >18.2 MΩ cm) was obtained from Easy (Heal Force, Shanghai, China) water purification systems and used to prepare the CPRG stock solution. Dimethyl sulfoxide (DMSO, Honeywell, Charlotte, NC, USA) was used for the preparation of fluorescein di(β-D-galactopyranoside) (FL). Filter disc containing frozen *Saccharomyces cerevisiae* strain BJ1991 yeast cells, minimal medium, solutions containing individually vitamins, L-aspartic acid, L-threonine, copper sulfate, and magnesium sulfate solutions were acquired from Xenometrix (Allschwil, Switzerland).

Bisphenol A, butylparaben, CPRG, Cytodex® 1 microcarrier beads (cross-linked dextran with positively-charged hydrophilic diethylaminoethyl exchanger groups at the surface, with approximately 190 μm in size), 1,4-dithiothreitol (DTT), 17α-ethynylestradiol (EE2), 17β-estradiol (E2), estrone (E1), FL, genistein, lyticase from *Arthrobacter luteus* (CAS number 37340-57-1, Ref. L2524), Triton™ X-100, and glycerol 87 % were purchased from Sigma Aldrich (St. Louis, MO, USA), and magnesium chloride was bought from Acros Organics (Geel, Belgium). Nonylphenol and Trizma® base were purchased form Honeywell (Charlotte, NC, USA). Hydrochloric acid 37 % and Dulbecco's phosphate buffered saline 10x without Ca^{2+} and Mg^{2+}, pH 6.8, containing potassium chloride 2 g L^{-1}, potassium phosphate monobasic anhydrous 2 g L^{-1}, sodium chloride 80 g L^{-1}, and sodium phosphate dibasic anhydrous 11.5 g L^{-1} (diluted 10x before use) were purchased from VWR Chemicals (Radnor, PA, USA).

To prepare the growth medium, reagents and solutions were used as supplied. Minimal medium (9.55 mL) was supplemented with vitamin solution (0.1 mL), L-aspartic acid solution (0.25 mL), L-threonine solution (0.08 mL), copper sulfate solution (0.025 mL), and magnesium sulfate solution (0.1 mL). Lysis buffer consisted in a solution of Tris-HCl (0.1 M, pH 7.8) containing 0.5 % (v/v) of Triton™ X-100, 20 mM of DTT, 1 mM of $MgCl_2$, and 0.1 g L^{-1} of lyticase.

2.2. Cell growth and maintenance

For growing yeast cells from the frozen state, the filter disk was placed in a T25 flask (Xenometrix or Corning, Corning, NY, USA) with 5 mL of growth medium for approximately 48 h at 31 °C with constant agitation at 65 rpm in an ES-60 incubator shaker (MIULAB, Hangzhou, Zhejiang, China). Before each assay, cells were diluted 1:10 with growth medium and incubated overnight in the same conditions. When not in use, yeast cells were frozen and stored at −80 °C in a solution containing 15 % glycerol for subsequent uses.

2.3. Implementation of fluorescent substrate

All fluorescence measurements were carried out in a Cytation™ 3 reader controlled by the Gen5 software (BioTek Instruments, Winoosky, VT, USA). The final concentration of the FL substrate implemented in this assay was fixed on 2.5 μM. When performing dilution of the yeast cells, optical density at 690 nm was assessed and the following equation was applied to normalise the initial concentration of cells:

$$Volume\ of\ yeast\ cells\ (mL)\ to\ 20\ mL\ of\ Growth\ Medium = \frac{0.6}{OD690} \quad (1)$$

For analysis, 80 μL of growth medium, 20 μL of EDC standard, and 100 μL of yeast cells suspension (with and without dilution to adjust the initial quantity) were added to each well and incubated for 6, 24, 30, 48, and 56 h. All the material used was sterile and the assay was performed under aseptic conditions. During incubation, the microtiter plate was covered with a gas-permeable plate sealer and placed in a closed box with moist paper, to ensure a humid atmosphere. After the incubation

period, 20 μL of growth medium containing FL were added and fluorescence was monitored for 15 min using $\lambda_{exc} = 460$ nm and $\lambda_{em} = 515$ nm. Each condition was tested in triplicate.

2.4. YES commercial kit

The XenoScreen YES (Xenometrix) is a commercially available yeast-based microplate assay for the detection of compounds with estrogenic agonistic activity, employing *S. cerevisiae* strain BJ1991 yeast cells. This assay kit was used as a comparison method for the analysis of samples, resorting to the alternative protocol present in the *Instructions for Use* provided by the supplier. In summary, assay medium was prepared by diluting the substrate CPRG in growth medium, and E2 was diluted in DMSO by performing half-log dilutions, followed by a 10-fold dilution with growth medium, with final concentrations ranging from $1.00\text{x}10^{-8}$ M to $1.00\text{x}10^{-11}$ M. Samples were subjected to the same 10-fold dilution step. Then, the initial quantity of yeast cells was normalised using Eq. (1). For analysis, 80 μL of assay medium, 20 μL of standard/sample, 20 μL of water (in the calibrators' wells) or 10 % DMSO solution (in the samples' wells), and finally 100 μL of yeast cells (with normalisation of the initial concentration of the inoculum) were added sequentially to each well of a non-binding transparent microtiter plate. After 48 h (or less, if the colour of the wells is sufficiently intense) the optical densities at 690 nm (for cellular growth) and at 570 nm (for β-galactosidase expression) were measured in a Cytation™ 3 reader and the induction ratio was calculated.

2.5. Yeast cells experimental parameters

Studies were performed by changing one variable at a time (OVAT), requiring a low quantity of yeast per experiment and generating a low amount of waste when there was any problem with the culture (e.g. contamination, slow growth). For the study on the lysis of yeast cells, 100 μL of growth medium, 20 μL of EDC standard, and 100 μL of yeast cells suspension (used directly or with dilution to normalise the initial quantity) were added to each well and incubated for 6, 13, and 19 h. The stock FL probe solution was diluted in lysis solution (final concentration in the well of 2.5 μM) and added to each well, following reaction monitoring for 1 h. For the experiments concerning the effect of the quantity of yeast cells, 100 μL of growth medium, 20 μL of EDC standard, and 100 μL of yeast cells suspension without initial quantity adjustment were incubated for 6 h at 31 and 34 °C in an ES-60 incubator shaker. Then, 50, 100, and 150 μL of the content of each well was transferred to another microtiter plate and a given volume of lysis solution was added to make up to 220 μL while maintaining the final concentration of FL at 2.5 μM. This procedure was replicated for the evaluation of the effect of the presence of a solid support, where yeast cells were grown overnight in the presence of Cytodex® 1 microcarrier beads at 3 and 6 mg mL^{-1}, reaching an optical density at 690 nm of ~0.6 before the assay. Each condition was tested in triplicate.

2.6. Preparation of microcarrier beads

Approximately 20 mg of dry Cytodex® 1 microcarrier beads were weighed and kept in Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS) for at least 3 h at room temperature with occasional gentle agitation in an Eppendorf ThermoMixer® C (Hamburg, Germany). Then, the suspension was centrifuged in a MiniSpin® centrifuge (Eppendorf) for 10 min at 12100×*g* and at room temperature, the supernatant was removed, and the solid residue was washed once with gentle agitation using the same buffer. After another centrifugation step, the supernatant was again discarded and replaced with fresh Ca^{2+}/Mg^{2+}-free PBS, to a final concentration of 20 mg mL^{-1}. The beads were then sterilized by autoclaving at 121 °C for 15 min and stored at 4 °C until use.

2.7. Microplate assay and analysis of samples

Water samples were collected before (sample #7) or after secondary wastewater treatment (samples #1–6) from a wastewater treatment plant located in the North of Portugal. Samples #1–3 were spiked to a concentration of E2 of $1\text{x}10^{-8}$ M and sample #1 spiked to a concentration of E2 of $1\text{x}10^{-9}$ M.

Initially, the EDCs were diluted in DMSO by performing half-log dilutions, followed by a 10-fold dilution with growth medium, with final concentrations ranging from $3.16\text{x}10^{-11}$ M to $1.00\text{x}10^{-8}$ M for E2, $1.00\text{x}10^{-11}$ M to $1.00\text{x}10^{-8}$ M for estrone, $3.16\text{x}10^{-12}$ M to $3.16\text{x}10^{-9}$ M for ethynylestradiol, $1.00\text{x}10^{-8}$ M to $3.16\text{x}10^{-6}$ M for nonylphenol, $1.00\text{x}10^{-7}$ M to $3.16\text{x}10^{-5}$ M for bisphenol A, $1.00\text{x}10^{-8}$ M to $1.00\text{x}10^{-5}$ M for butylparaben, and $3.16\text{x}10^{-8}$ M to $1.00\text{x}10^{-5}$ M for genistein. Samples were subjected to the same 10-fold dilution step. Then, to each well, 80 μL of growth medium, 20 μL of the standard/sample, 20 μL of water (in the calibrators' wells) or 10 % DMSO solution (in the samples' wells), and finally 100 μL of yeast cells (without normalising the initial concentration of the inoculum) were added sequentially. After the incubation period (2, 4, or 6 h), 100 μL of the content of each well were transferred to a different microtiter plate, 120 μL of lysis solution containing FL were added, and the reaction was monitored during 1 h. Each sample was analysed in triplicate in three different days.

2.8. Statistical analysis

The lower limit of quantification (LLOQ) is defined by the European Medicines Agency as the lowest amount of an analyte in a sample that can be quantitatively determined, corresponding to the lowest calibration standard. EC10 (the concentration that yielded a signal of 10 % of the maximum value) corresponds to the threshold for the classification of a sample as having agonistic estrogenic activity. Interpolation of analytical signals, EC50 (the concentration that yielded a signal of 50 % of the maximum value), and EC10 ($n \geq 3$) were calculated using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Implementation of fluorescent substrate

The commercial YES kit standard protocol contemplates the addition of the enzymatic substrate when inoculating the assay plate, so that the output of the photometric probe can occur from the moment the expressed enzyme is released from the yeast to the extracellular media. Hence, the first experiment with the fluorimetric probe was performed similarly. The results show that the fluorescent moiety of the substrate was released, since the fluorescence values were high, however there were no differences between the fluorescence emission in the blanks, where no E2 was present, and the standards (data not shown). This could have happened due to the hydrolysis of the substrate. To circumvent this possibility, the addition of the substrate was only performed immediately before reading the plate, after the incubation of yeast with EDC compounds or samples.

Yeast cells were added to the wells at two different amounts, one with the initial quantity adjusted according to OD690 (Eq. (1)), and the other without any dilution, directly from the T25 incubation flask. The curves obtained for the highest concentration of E2 ($1.00\text{x}10^{-8}$ M) are shown in Fig. 1, with a similar trend for all the other tested concentrations.

At the lowest incubation time – 6 h, there was no difference between blanks and positive controls and, until 30 h of incubation, the inoculum without normalisation of yeast quantity presented a better response than the normalised one. However, at 48 and 56 h, diluted yeasts presented higher fluorescence emission, probably because there was less competition for nutrients and less metabolic products released to the medium

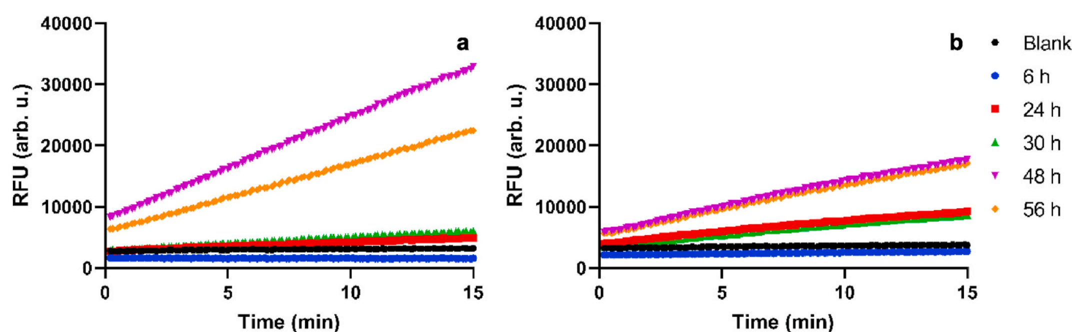

Fig. 1. Results obtained with yeast cells with and without inoculum adjustment after different incubation times, namely 6, 24, 30, 48, and 56 h with 1.00x10^{-8} M of E2 at 31 °C. Graph (**a**) represents the normalised yeast initial inoculum and graph (**b**) represents yeasts without quantity adjustment using 2.5 μM of FL substrate. The represented blank concerns the 56 h assay, which is considered the worst-case scenario for background signal, having other incubation times attained similar or lower blank values.

that would impair yeast growth. Since one of the goals of this work is to decrease the incubation time, the initial concentration normalisation seems to hinder this objective.

3.2. Lysis of yeast cells

The next experiment consisted in evaluating the effect of adding a lysis solution to each well, to improve the release of β-galactosidase, fostering detection after shorter incubation times. The curves obtained for the highest concentration of E2 (1.00x10^{-8} M) are shown on Fig. 2.

For most of the tested conditions, the results show an exponential curve where in the first part the fluorescence increase is slow in the first 20 min. This initial profile is probably related to the degradation of the yeast cell wall by the lytic enzyme, with the release of the intracellular beta-galactosidase. Except for the experiment with normalisation of inoculum and the lowest incubation time (6 h), the second part of the curves shows an exponential increase ($R^2 > 0.99$) in the fluorescence emission, related to the enzymatic cleavage of the substrate and release of the fluorescent moiety. It is observed that the higher the concentration of yeast cells, the shorter incubation period is needed, which supports the previously obtained results. However, for longer incubation periods (e.g. 13, 19 h), the amount of yeast cells is not relevant. When comparing the results obtained with lysis of the yeast cells with those without lysis, a decrease of assay time was observed since the evaluation of enzymatic activity was possible after 6 h of incubation. In Fig. S2 the lysis of yeast cells by microscopy can be observed. In images a, b, and c of Fig. S2, with small adjustments on the fine focus knob, different planes of the beads are represented, showing the presence of yeast cells. In image d, the effect of cellular lysis is demonstrated since no yeast cells are visible.

3.3. Volume of yeast cells suspension and incubation temperature

After establishing that 6 h of incubation were sufficient to assess the enzymatic induction (β-galactosidase expression) when the yeast lysis step was added, the volume of yeast cells (with no initial inoculum normalisation) and incubation temperature were evaluated. The curves obtained for the highest concentration of E2 (1.00x10^{-8} M) at 31 and 34 °C are shown on Fig. 3.

The increase in the incubation temperature was detrimental to the sensitivity of the assay, because 31 °C is the optimal growth temperature for this *S. cerevisiae* strain. When comparing the different yeast suspension volumes subjected to the lysis procedure, 100 μL provided the highest sensitivity of all tested conditions, with fluorescence values approximately 1.7-fold higher compared to 150 μL. Additionally, 50 μL were not sufficient to elicit a measurable signal. Therefore, the volume of 100 μL was chosen for further experiments.

3.4. Use of a solid support

It was hypothesised that the presence of a solid support, namely microcarriers beads, could improve the assays' sensitivity, by allowing for a larger surface for yeast cells to grow on, similarly to what has been described with animal cells [29]. Hence, the effect of the presence of Cytodex® 1 on the growth stage of the yeast cells, before exposure to compounds/samples with estrogenic activity, was assessed using two different concentrations of particles, 3 and 6 mg mL^{-1}. The curves obtained for 100 μL of yeast cell suspensions with and without beads, exposed to the highest concentration of E2 (1.00x10^{-8} M) after 6 h incubation at 31 °C, are shown on Fig. 4.

The results show an increase in the assay sensitivity when the yeast cells are grown overnight with Cytodex® 1 microcarrier beads, being the effect more noticeable for the highest concentration of beads. This

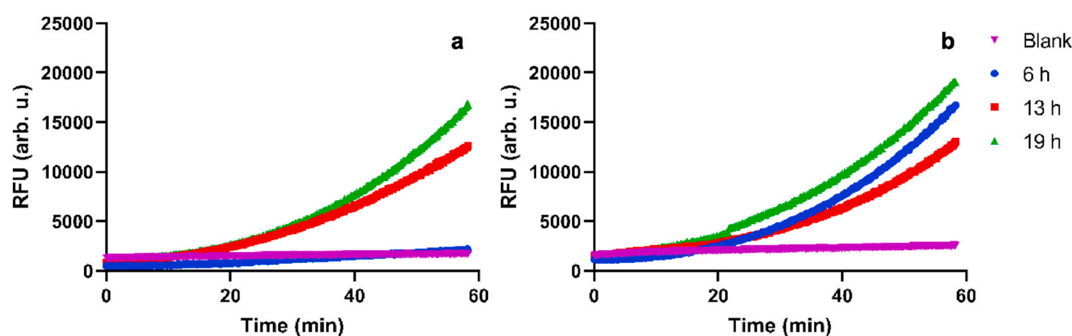

Fig. 2. Results obtained with yeast cells with (**a**) and without (**b**) normalisation of the quantity in the initial inoculum, after different incubation times with 1.00x10^{-8} M of E2 at 31 °C, including a lysis step before starting fluorescence monitoring, and using 2.5 μM of FL substrate. The represented blank concerns the 19 h assay, which is considered the worst-case scenario for background signal, having other incubation times attained similar or lower blank values.

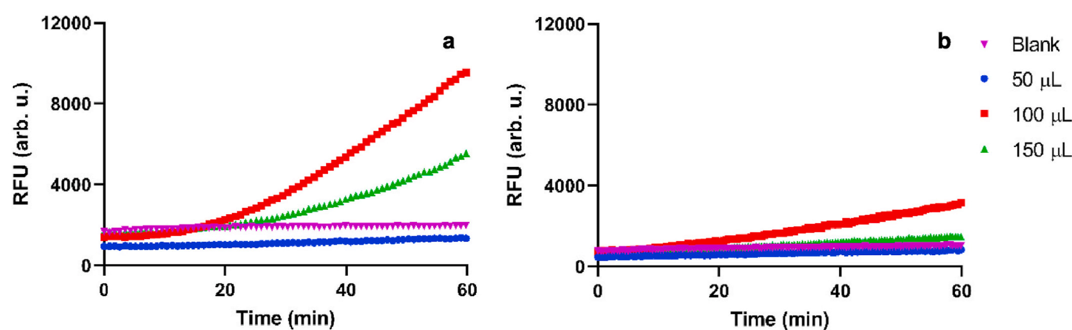

Fig. 3. Results obtained for different volumes of yeast cells suspension incubated at 31 °C **(a)** and 34 °C **(b)** for 6 h with 1.00×10^{-8} M of E2 and 2.5 μM of FL substrate. The represented blank concerns the assay using 150 μL, which is considered the worst-case scenario for background signal, having other incubation volumes attained similar or lower blank values.

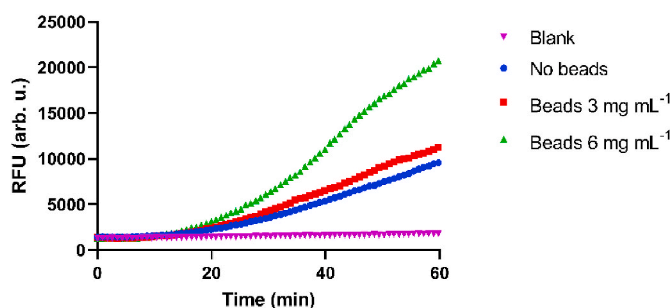

Fig. 4. Effect of the use of Cytodex® 1 microcarrier beads during yeast cells growth phase on the assay's sensitivity, at 6 h of incubation with 1.00×10^{-8} M of E2 at 31 °C and using 2.5 μM of FL substrate. The represented blank concerns the assay using microcarrier beads at 6 mg mL^{-1}, which is considered the worst-case scenario for background signal, having the other conditions attained similar or lower blank values.

indicates that the beads provided an increased surface for cellular growth and, when in the presence of a higher concentration of beads, a higher yeast cell mass is available for the lysis procedure, therefore more enzyme is released to the medium and can catalyse the breakdown of the fluorescent substrate. To confirm that the results obtained in the previous section (*Volume of yeast cells suspension and incubation temperature*) were replicable concerning the ratio yeast volume:lysis solution when using a solid support, this experiment was also performed by subjecting 150 μL of yeast cells grown overnight with Cytodex® 1 microcarrier beads to the lysis step. The fluorescent emission using 100 μL of yeast cells in microcarriers was approximately 2-fold higher than that using 150 μL (data not shown), confirming the results obtained previously. Fig. S2 shows the growth of yeast cells in the medium as well as on the surface of the microcarrier beads where, by adjusting the microscopic focus plane, yeast cells are clearly visible on the edges of the beads and on top of them, demonstrating their growth on the microcarrier surface, taking advantage of the higher surface area available.

3.5. Reduction of the assay time

The previous results have shown that measurable results can be attained in only 6 h, and this would be a major improvement compared to the standard assay kit. Nevertheless, shorter incubation times of 2 and 4 h were evaluated to assess if assay time could be further reduced, while maintaining the analytical performance, namely EC50 values, based on the estrogenic effect induced by E2. For 2 h of incubation, EC50 values was 0.17 ± 0.03, while it was 0.29 ± 0.05 for 4 h and 0.36 ± 0.03 nM for 6 h. LLOQ values were the same, corresponding to 0.03 nM. These values demonstrate that lower EDC values can be detected after 2 h of incubation, when compared to longer periods, even though the amplitude of the fluorescent signal (assay sensitivity) is smaller. These figures are comparable with the ones from the standard assay kit for measurements taken after 45 h of incubation, which provided an EC50 of 0.35 ± 0.09 nM. They are also in the same order of magnitude of other published works using *S. cerevisiae*-based detection, at < 1 nM for the EC50 values for E2 [13,14,23,27,30].

The results attained for all the tested EDCs after 2 h of incubation are presented in Table 1 and compared with previously reported data from the colorimetric YES assay and with alternative approaches like *S. cerevisiae*-based bioluminescent assays or the E-Screen assay, which uses human breast cancer cells. The dose-response curves obtained for the different EDCs are presented in Fig. 5, while raw data is available in Tables S1 and S2.

These results show that the proposed method provided similar results for 17α-ethynylestradiol when compared with the reported results for the colorimetric YES assay. It also presented decreased EC50 values for the analysis of E2, estrone, bisphenol A, butylparaben, and nonylphenol, which is translated in the detection of these EDCs at lower levels. Additionally, when comparing with other alternative approaches employing *S. cerevisiae*, the proposed method attains comparable or improved results for EC50 in all compounds except for genistein. Only for E2, 17α-ethynylestradiol, and estrone, the EC50 values of methods that use different cell lines, like the E-Screen (human breast cancer cells) are lower when compared with the method here described. However,

Table 1
EC50 values obtained for all tested EDCs and comparison with published data.

Compound	EC50 (nM)		
	Present work	Previously reported YES values	Alternative methods
17β-estradiol	0.17 ± 0.03	0.35[a] – 0.44 [19,28]	0.0053 [19] – 0.6 [14]
17α-ethynylestradiol	0.16 ± 0.06	0.17 [28] – 0.56 [19]	0.011 [19] – 1.2 [14]
Estrone	0.5 ± 0.2	1.8 [28] – 2.5 [19]	0.082 [19] – 137 [24]
Bisphenol A	$(1.2 \pm 0.1) \times 10^3$	4.9×10^3 [28]	345 [27] – 2.8×10^3 [28]
Butylparaben	$(3.2 \pm 0.3) \times 10^2$	8.3×10^3 [19]	5.9×10^2 [19] – 1.0×10^3 [24]
Genistein	$(5.4 \pm 0.6) \times 10^2$	N.A.	106 [24] – 153 [27]
Nonylphenol	101 ± 4	4.2×10^3 [19]	5.5×10^3 [19] – 1.7×10^4 [28]

[a] Result attained *in house* with a commercial colorimetric assay kit; N.A. – Not available.

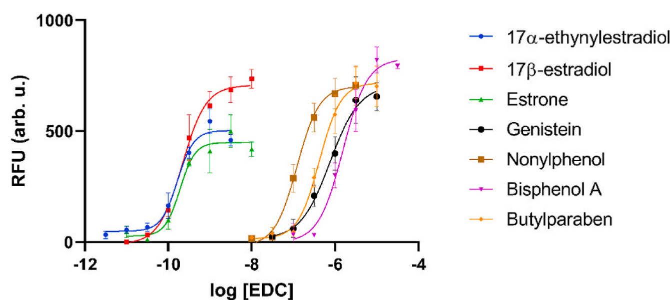

Fig. 5. Dose-response curves obtained for all tested EDCs after 2 h of incubation.

Table 2
Results of agonistic estrogenic activity obtained with the present method and with the commercial assay kit.

Sample	Agonistic estrogenic activity?	
	Present method	Commercial assay kit
Sample #1	No	No
***Sample #1 spiked* 1.0 nM**	No	No
***Sample #1 spiked* 10 nM**	Yes	Yes
Sample #2	No	No
***Sample #2 spiked* 10 nM**	Yes	Yes
Sample #3	No	No
***Sample #3 spiked* 10 nM**	Yes	Yes
Sample #4	No	No
Sample #5	No	No
Sample #6	No	No
Sample #7	No	No

these cell lines are more difficult to cultivate and maintain than *S. cerevisiae* yeasts.

The response to certain EDCs, such as bisphenol A, butylparaben, and genistein, occurs for higher concentration of these compounds compared to E2, showing their lower potencies as endocrine disruptors in the model system. Nevertheless, the improvement of the EC50 values when compared to previously reported works is important. This may allow to better evaluate synergistic or additive biological effects of different EDCs, since environmental samples often contain mixtures of contaminants that can modify the extent of the biological effect compared to when only one compound is present.

3.6. Analysis of samples

To validate the applicability of the assay, wastewater samples (including some spiked with E2) were screened by the proposed method and the results compared with the commercial assay kit. A sample is considered to have agonistic estrogenic activity if the signal is $>$ EC10. The results obtained with both methods are indicated in Table 2.

Therefore, the present method yielded the same results in terms of agonistic estrogenic activity when compared with the commercially available kit, after only 2 h of exposure to the inductor, in contrast with the 45 h of the commercial assay kit. When considering the samples with agonistic estrogenic activity, the determined E2 concentrations (spike level of 10 nM) are 8 ± 4 nM for sample #1, 12 ± 3 nM for sample #2, and 11.0 ± 0.7 nM for sample #3, showing acceptable recovery values (80–120 %).

4. Conclusion

The present work shows the development of a high-throughput methodology for the assessment of EDCs in water samples. The incubation/chemical challenge step was reduced to only 2 h, while maintaining the sensitivity on pair with other approaches employing *S. cerevisiae*. The use of microcarrier beads increased the surface on which the yeast cells could grow while the lysis step fostered the release of the intracellular enzyme, with a positive impact on the assay's sensitivity. When considering the experimental preparation of the yeast cells, only dilution of the initial suspension and addition to the solid support was required, providing a simple and straightforward procedure. As a commercially available modified strain of *S. cerevisiae* is employed, expertise on synthetic biology and metabolic engineering tools is not required for its implementation.

When analysing samples from different wastewater treatment stages, the obtained results agreed with those found using a commercially available assay kit, validating the proposed approach, with an improvement in the EC50 values, as well as in the assay time. Recovery values are comprised between 80 and 120 % and the proposed approach is intended to be used as a semi-quantitative evaluation. This is important considering that possible interactions between EDCs present in the sample or between EDCs and matrix components may alter the biological response. Likewise, the proposed method can be used with complex water matrices after just a filtration step, possessing the merits to be used routinely for the evaluation of the presence of EDCs in water samples. For a full characterization of the composition of each sample, the data collected through this method can be conjugated with information attained from other techniques, such as HPLC-MS/MS. This approach presents the opportunity for automation, and it is envisaged as future work.

CRediT authorship contribution statement

Bruno J.R. Gregório: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Writing – original draft, Visualization, Writing – review & editing. **Inês I. Ramos:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Sara S. Marques:** Investigation, Resources, Writing – review & editing. **Luísa Barreiros:** Investigation, Methodology, Resources, Writing – review & editing. **Luís M. Magalhães:** Conceptualization, Writing – review & editing. **Rudolf J. Schneider:** Conceptualization, Writing – review & editing. **Marcela A. Segundo:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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