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Rat cardiomyocyte H9c2(2-1)-based sulforhodamine B assay as a promising *in vitro* method to assess the biological component of effluent toxicity

Elsa T Rodrigues[1,*], *Susana F Nascimento*[2], *Maria João Moreno*[2], *Paulo J Oliveira*[3], *Miguel A Pardal*[1]

[1] *University of Coimbra, Centre for Functional Ecology, Department of Life Sciences, Calçada Martim de Freitas, Coimbra 3000-456, Portugal*

[2] *University of Coimbra, Coimbra Chemistry Center, Department of Chemistry, Coimbra 3004-535, Portugal*

[3] *Centre for Neuroscience and Cell Biology, University of Coimbra, UC Biotech, Biocant Park, Cantanhede 3060-197, Portugal*

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ABSTRACT

The treatment of wastewaters is crucial to maintain the ecological status of receiving waters, and thereby guarantee the protection of aquatic life and human health. Wastewater quality evaluation is conventionally based on physicochemical parameters, but increasing attention has been paid to integrate physicochemical and biological data. Nevertheless, the regulatory use of fish in biological testing methods has been subject to various ethical and cost concerns, and *in vitro* cell-based assays have thus become an important topic of interest. Hence, the present study intends: (a) to evaluate the efficiency of two different sample pre-concentration techniques (lyophilisation and solid phase extraction) to assess the toxicity of municipal effluents on rat cardiomyoblast H9c2(2-1) cells, and (b) maximizing the use of the effluent sample collected, to estimate the environmental condition of the receiving environment. The gathered results demonstrate that the H9c2(2-1) sulforhodamine B-based assay is an appropriate *in vitro* method to assess biological effluent toxicity, and the best results were attained by lyophilising the sample as pre-treatment. Due to its response, the H9c2(2-1) cell line might be a possible alternative *in vitro* model for fish lethal testing to assess the toxicity of municipal effluents. The physicochemical status of the sample suggests a high potential for eutrophication, and iron exceeded the permissible level for wastewater discharge, possibly due to the addition of ferric chloride for wastewater treatment. In general, the levels of carbamazepine and sulfamethoxazole are higher than those reported for other countries, and both surpassed the aquatic protective values for long-term exposure.

Introduction

It is well known that wastewater has a direct impact on the biological diversity of aquatic ecosystems, being its treatment essential to maintain the ecological status of the receiving waters, and thereby guarantee the protection of aquatic life and human health. Wastewater quality evaluation is conventionally based on physicochemical parameters, but increasing attention has been paid to integrate physicochemical and biological data in order to better evaluate the possible hazard and risk for the aquatic life (*e.g.*, OSPAR Convention, 2000), and fish

* Corresponding author.
E-mail: etrodrig@uc.pt (E.T. Rodrigues).

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representing aquatic vertebrate organisms are largely used in a standard 96-h exposure test according to international guidelines (*e.g.*, ASTM E1192-97, 2014). However, the use of fish in biological testing methods has been subject to various ethical and cost concerns (*e.g.*, European Commission, 2010), and thus *in vitro* cell-based assays have become an important topic of interest as possible alternative. A pre-treatment step of effluent sample concentration could overcome the limitation of cell sensitivity attributed to cell-based assays when compared in absolute terms to fish testing (*e.g.*, Schirmer, 2006), as well as increase the sensitivity of cell lines through cell culture selection. Hence, lyophilisation or solid phase extraction (SPE) are considered suitable pre-concentration techniques (*e.g.*, Bernhard et al., 2017) and, as was previously shown, rat cardiomyoblast H9c2(2-1) cells presented the highest sensitivity, when compared with other mammalian and/or fish cell lines, to pesticides (Rodrigues et al., 2015, 2019), pharmaceuticals (Rodrigues et al., 2020) and toxins (Neves et al., 2020; Varela et al., 2020), and the same is corroborated by Bains et al. (2013) also for pharmaceuticals, by Han et al. (2017) for industrial chemicals, and by Sumi et al. (2011) for arsenite. Originally derived from embryonic left ventricular rat heart tissue (Kimes and Brandt, 1976), the H9c2(2-1) cell line is commonly used as an experimental model to study the effects of environmental pollutants since there is a relationship between these compounds and cardiac hypertrophy (*e.g.*, Huang et al., 2016; Selmin et al., 2005) .

Two well-defined objectives were delineated for the present study: The first aims to evaluate the efficiency of two different sample pre-concentration techniques, lyophilisation and SPE, to assess the toxicity of a municipal effluent on H9c2(2-1) cells as a preliminary study with a filtered (0.22 µm) effluent sample directly prepared in Dulbecco's modified Eagle's medium (DMEM)-high glucose cell culture medium which considered the adjustment of its growing medium constituents as a means to overcome sample dilution effect was carried out with no cytotoxic results (data not shown). The second uses the collected effluent sample to estimate the environmental condition of the receiving environment by comparing measured physicochemical parameters with the established parametric values.

The effluent sample (outlet effluent) was collected in the wastewater treatment plant of Choupal (Coimbra, Portugal), which is responsible for the treatment of the effluent of 143,396 residents (Portuguese 2011 census). The Choupal wastewater treatment plant has a treatment capacity for 213,000 people (corresponding to a daily average flow of 36,000 m^3), using a decanter as the primary treatment process, and a secondary treatment of trickle beds. Whilst municipal wastewater is known to be the main source of the phosphorus load which reaches surface waters (Mockler et al., 2017), its contribution to other contaminant loads of environmental concern, as pharmaceuticals, is less well recognised and has been the subject of recent studies worldwide (Fekadu et al., 2019; Paíga et al., 2019; Palli et al., 2019; Rivera-Jaimes et al., 2018; Zhang et al., 2018). Therefore, a physicochemical characterization of the effluent sample was also performed aiming to compare the results with the parametric values established in the Portuguese legislation for environmental protection against effluent disposal in the water environment (Decree Law 236/98 (1998), *Annex XVIII*). As Coimbra hosts the Coimbra Hospital and Universitary centre, the biggest Portuguese hospital with integrated research and a teaching and patient care approach with a total number of beds of 2038 (in 2014), the presence of pharmaceutical chemicals in the river Mondego, the receiving waters, is a matter of concern. Therefore, the neuroactive compound carbamazepine (CBZ, CAS 298-46-4) and the antibiotic sulfamethoxazole (SMZ, CAS 723-46-6) were also investigated in the effluent sample as an example of the possible drug contamination of wastewaters. In this regard, an analytical method was developed and validated for the simultaneous determination of both human-use pharmaceuticals. Both drugs are in the Top 20 of a global-scale analysis concerning the presence of 203 pharmaceuticals in freshwater ecosystems across 41 countries (Hughes et al., 2013). Also, in a review study, they were both considered, with other 16 pharmaceutical compounds, the most frequent and persistent pharmaceuticals found in effluent samples, as well as those with the highest environmental risk (Verlicchi et al., 2012), and CBZ and its metabolites 10,11-dihydro-10,11-dihydroxy-CBZ, 10,11-dihydro-10-hydroxy-CBZ, 10,11-epoxy-10,11-dihydro-CBZ, 2-hydroxy-CBZ and 3-hydroxy-CBZ were also considered very persistent with little to no removal during wastewater treatment (Bahlmann et al., 2014). Moreover, they were both determined in an hourly variation study of 83 pharmaceuticals sampled in effluents discharged by a wastewater treatment plant of Leiria, also in Portugal (Paíga et al., 2019).

Besides contributing to develop an alternative *in vitro* method to assess the biological component of effluent toxicity, as well as for the implementation of the 3Rs principle of *replacement, refinement* and *reduction* animal testing, the gathered results could also provide important information regarding the cytotoxicity of a municipal effluent and allow to evaluate in a more comprehensive and realistic approach its possible impact on the water quality of the receiving environment and its potential effects on aquatic organisms.

1. Material and methods

1.1. Site description and sampling procedures

The effluent sample was collected once in the discharge point of the wastewater treatment plant of Choupal (40°13′13.08″N, 8°27′6.12″W) using a sampler with a telescopic rod (Bürkle 331-2131). During collection (May 30, 2018), temperature (°C), electrical conductivity (µS/cm), pH and dissolved oxygen (%) was measured in the sample (7.0 L) using a multi-parameter portable meter (WTW 3320 SET-1). Immediately after arrival at the laboratory (≈20 min), 150 mL of the sample was acidified to pH below 2 with HNO_3 (65% concentrate, Panreac 213255) and stored in a glass container for metal determinations (Table 1), and triplicated blanks were included in the sample batch for analytical quality control. The four glass containers were left overnight with HNO_3 (65% concentrate), and were thoroughly rinsed with ultra-pure water and dried before sample storage. Chemical oxygen demand (COD), total suspended solids (TSS), dissolved silicon and silicon dioxide, total phosphorus, dissolved nitrates and ammonium were also determined. For the determination of COD, TSS, total phosphorus and ammonium, 2 × 500 mL of effluent were stored in PET (polyethylene terephthalate) containers. Then, the remaining sample was filtered through a 0.45 µm pore size membrane and stored for determination of dissolved silicon and silicon dioxide (PET container) and nitrates (PET container). Moreover, for cell-based assays, filtered samples (80 and 150 mL) were deep-frozen (-80 °C) in glass containers for further lyophilisation (Unicryo MC-4 L), as well as refrigerated (500 mL at 4 °C) in a glass container for SPE (see Section 1.2). In order to minimize the microbial degradation of the sample, SPE procedures were performed within 48 h of sample collection. Glass containers were selected for SPE sample storage and used in the cell-based assays as the municipal effluent tested is expected to have more organic than inorganic toxic compounds. Sub-samples were stored/preserved as reported in Table 1.

1.2. Solid phase extraction method

According to US-EPA 1694 (2007), Oasis HLB cartridges (Waters 186000116) were selected to develop the SPE method.

Table 1 – Chemical characterization: sub-sample storage, expression of the results and reference of the method/kit used for each analysed parameter.

	Sub-sample storage	Expression of results	Method/Kit
Chemical oxygen demand	frozen	mg/L O_2	PTE 13 (20.10.2015), test laboratory accredited
Total suspended solids	4 °C	mg/L	Gravimetric method (see Section 2.4)
Dissolved silicon	4 °C	mg/L Si	kit Nanocolor Test (Macherey-Nagel Test 1–48)
Dissolved silicon dioxide	4 °C	mg/L SiO_2	kit Nanocolor Test (Macherey-Nagel Test 1–48)
Total phosphorus	frozen	mg/L P	MI (EAM: EPA 365.2), test laboratory not accredited
Dissolved nitrates	frozen	mg/L NO_3	PTE 56 (22.03.2017), test laboratory accredited
Ammonium	frozen	mg/L NH_4	PTE 26 (12.12.2016), test laboratory accredited
Total arsenic	pH ≤2, 4 °C	mg/L As	Inductively Coupled Plasma Optical Emission
Total cadmium	pH ≤2, 4 °C	mg/L Cd	Inductively Coupled Plasma Optical Emission
Total chromium	pH ≤2, 4 °C	mg/L Cr	Inductively Coupled Plasma Optical Emission
Total cobalt	pH ≤2, 4 °C	mg/L Co	Inductively Coupled Plasma Optical Emission
Total copper	pH ≤2, 4 °C	mg/L Cu	Inductively Coupled Plasma Optical Emission
Total iron	pH ≤2, 4 °C	mg/L Fe	Inductively Coupled Plasma Optical Emission
Total lead	pH ≤2, 4 °C	mg/L Pb	Inductively Coupled Plasma Optical Emission
Total manganese	pH ≤2, 4 °C	mg/L Mn	Inductively Coupled Plasma Optical Emission
Total mercury	pH ≤2, 4 °C	mg/L Hg	Cold Vapour Atomic Fluorescence Spectroscopy
Total nickel	pH ≤2, 4 °C	mg/L Ni	Inductively Coupled Plasma Optical Emission
Total zinc	pH ≤2, 4 °C	mg/L Zn	Inductively Coupled Plasma Optical Emission
Carbamazepine	4 °C*	µg/L CBZ	HPLC-DAD
Sulfamethoxazole	4 °C*	µg/L SMZ	HPLC-DAD

*analysis within 48 hr of sample collection.
HPLC-DAD, high performance liquid chromatography coupled with a diode array detector.

Cartridges pre-conditioned with 5.0 mL of methanol (MeOH, ChromasolvV™Gradient for HPLC, ≥99.9%, Riedel-de Haën 34885) plus 5.0 mL of deionized water, with a flow-rate of 1.0 mL/min were used to load the samples at a flow-rate of 10 mL/min. After this loading step, the cartridge was rinsed with 5.0 mL of deionized water for the removal of interfering substances, and then completely dried under vacuum (≈15 min) (Gros et al., 2006). Finally, the compounds were eluted using 8.0 mL of MeOH (4 × 2.0 mL) at a flow-rate of 1.0 mL/min. The SPE was performed with 80 mL of filtered effluent sample for cell-based assays (named 80 mL SPE), as well as with 500 mL of filtered effluent sample, where the final SPE extract was divided in two 4.0 mL sub-extracts, representing each 250 mL of the original effluent sample. One sub-extract, named 250 mL *SPE*, was used to prepare the working solution for cell-based assays (see Section 1.3) and the other for CBZ and SMZ determinations (see Section 1.4). The SPE was also successively performed for the five standard concentrations used to determine extraction SPE method recoveries, and for a spiked effluent sample used for quality assessment. At the end, all the obtained SPE extracts were completely dried in a rotary evaporator (BUCHI Rotavapor R-114).

1.3. *Cell-based toxicity assessment*

1.3.1. *H9c2(2-1) cell culture*

H9c2(2-1) cells were purchased from American Type Culture Collection (CRL-1446) and grown as an adherent monolayer in filtered (Autofil 1102-RLS) DMEM-high glucose (Sigma-Aldrich D5648) culture medium adjusted to contain 1.8 g/L of sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS, Gibco 10270-106) and 1% antibiotic-antimycotic (Gibco 15240-062) at pH 7.3. They were, then, subcloned at a 70% confluence using a phosphate-buffered saline (PBS) solution to wash the cells and 0.05% trypsin-EDTA (Gilco 25300-062) to detach them from the culture flask (VWR 734-2313). H9c2(2-1) cells were incubated in a humidified atmosphere with 5% of CO_2 at 37 °C (CO_2 Unitherm, UniEquip).

1.3.2. *Sulforhodamine B assay*

The biological response was evaluated by determining H9c2(2-1) mass decrease using the sulforhodamine B (SRB) colorimetric assay. This assay was established by Skehan et al. (1990) and optimized by Vichai and Kirtikara (2006), and it has been vastly used to successfully evaluate H9c2(2-1) mass decrease exerted by environmental pollutants such as pesticides (Rodrigues et al., 2015, 2019), pharmaceuticals (Rodrigues et al., 2020) and toxins (Neves et al., 2020; Varela et al., 2020). The H9c2(2-1) cells were seeded (500 µL at 10^4 cells/mL density) in 48-well plates (Corning 3548) and allowed to attach 24 hr prior to the cell-based assay. All the sub-samples, which resulted from lyophilisation or SPE pre-treatments (see Table 2), were reconstituted in 3.7 mL of DMEM-high glucose culture medium to prepare the working solutions. To start the assays, 400 µL of the 500 µL well medium corresponding to the most concentrated well was removed and replaced with 900 µL of the working solutions (10% dilution), producing 19.4-, 36.5- and 61.8-fold concentrations related to the 80, 150 and 250 mL original effluent sub-samples, respectively. Then, starting from the most concentrated well, two-fold dilution series were applied to prepare the exposure solutions in a total of five concentrations (Table 2). Three independent experimental replicates were maintained for each concentration, and eight negative controls (cells with culture medium alone) were considered by replicate. In addition, in the 150 mL lyophilised assay (final assay used to confirm that sample lyophilisation was the best concentration pre-treatment), eight positive controls

Table 2 – Experimental conditions for H9c2(2-1) sulforhodamine B assays.

Pre-treatment	Passage used	Concentrations tested (%)
80 mL lyophilised*	#15	122/243/486/972/1944
150 mL lyophilised**	#14	228/456/913/1825/3650
80 mL SPE*	#15	122/243/486/972/1944
250 mL SPE*	#15	386/773/1545/3090/6180

*Assays used to select the best concentration pre-treatment.
**Assay used to confirm that sample lyophilisation is the best concentration pre-treatment.

(2% DMSO) were also considered by replicate. After a 24-hr exposure time, cells were washed with PBS and dried, and then fixed at -18 °C with cold 1% of acetic acid prepared in MeOH. Fixative was then removed and cells were stained for 60 min at room temperature using a SRB solution (prepared in 1% of acetic acid in ultra-pure water) and excess dye was removed by washing the wells at least four times with 1% of acetic acid in ultra-pure water. Protein-bound dye was dissolved under gentle stirring using 10 mmol/L Tris/base (Sigma-Aldrich T1503) at pH 10, and quantified from absorbance measurements (545 nm) using a microplate reader (BioTek Synergy HT). Absorbance data were used to determine EC_{50} results, which is a widely-recognised method to quantitatively relate toxic response to exposure and is very often used for comparative purposes. In the present study, toxicity EC_{50} data were calculated based on the initial volume percent of the effluent in the exposure solution, according to the international guideline ASTM E1192-97 (2014).

1.3.3. *Validity criteria and statistical analysis*

The coefficient of variation of the mean (CV, in percentage) was calculated for the negative controls to ascertain reproducibility and as plate acceptance criterion, which is CV ≤20% (Iversen et al., 2012). CV was calculated by the equation:

$$CV = (SD/\sqrt{N})/\text{arithmeticmean} \times 100\%$$

where SD is the standard deviation and *N* the number of negative control wells per independent experiment.

One-way analysis of variance followed by multiple comparisons Dunnett's test were used to detect statistically significant toxicity differences between the controls and effluent concentrations tested, and between controls and positive controls in the 150 mL lyophilised assay. ANOVAs were used after testing the assumption of normality by the Shapiro-Wilk test and homogeneity of variances by the Bartlett's test. A *P*-value of 0.05 was used as the cutoff for significance. These analyses were carried out using the STATISTICA Software version 7.

To determine EC_{50s} and 95% confidence intervals (CI), the results of the SRB assays (absorbance data) were expressed as a fraction of the controls. Then, a four-parameter logistic regression after log-transformation of *x*-axis values (concentration percentage of the sample) was applied. These analyses were carried out using the GraphPad Prism Software version 6. For valid assays, the fitted concentration-response curves should have a r^2 (coefficient of determination) >0.850, and the percent fitting error (%FE) of EC_{50} should be <40% (Beck et al., 2017). FE was calculated by the equation:

$$\%FE = FE(LogEC_{50}) \times Ln(10) \times 100\%$$

where FE ($LogEC_{50}$) is the standard error of $LogEC_{50}$.

1.4. *Chemistry-based assessment*

Several parameters were determined in order to chemically characterize the effluent sample (Table 1). TSS, dissolved silicon and silicon dioxide (analysed in triplicate) and the two pharmaceuticals were determined in our laboratories. For TSS determination, a well-mixed measured volume (275 mL) of the effluent sample was filtered through a pre-weighed glass microfiber filter (Whatman 1822-047). The filter was heated to constant mass at 105 ± 1 °C and then reweighed. The mass increase divided by the filtered water volume was equal to the TSS concentration. Metals were kindly determined by Prof. Eduarda Pereira's research team in the Chemical Department of the University of Aveiro (Portugal) using the methods presented in Table 1, and all the other parameters were obtained by outsourcing (COD, total phosphorus, nitrates and ammonium).

Table 3 – Summary of the linear gradient applied to the HPLC system for separation of the target analytes.

Time (min)	Eluent A (%)	Eluent B (%)
0	15	85
3	15	85
25	95	5
32	95	5
37	15	85
52	15	85

Eluent A, acetonitrile:MeOH (2:1, *V/V*).
Eluent B, 5.0 mmol/L of ammonium acetate (pH 4.7 with acetic acid).

A direct injection of SPE reconstituted extracts in MeOH:deionized water (1.0 mL for standards and the spiked effluent sample, and 0.5 mL for the effluent sample, 25:75, *V/V*) on a high performance liquid chromatography (HPLC) system (Agilent 1100 series G) coupled with a diode array detector (DAD, Agilent 1200 series G1315D) were used to separate and detect the pharmaceuticals CBZ and SMZ according to Gros et al. (2006), with minor modifications. Briefly, LC separations were performed in 52 min using a Zorbax ODS C_{18} (250 × 4.6 mm, 5 µmol/L) column and a Zorbax ODS C_{18} (12.5 × 4.6 mm, 5 µmol/L) pre-column, and a linear gradient of acetonitrile (HPLC gradient grade, Fisher Scientific A/0627/17):MeOH (2:1 *V/V*) as Eluent A and 5.0 mmol/L of ammonium acetate (pH 4.7 with acetic acid) as Eluent B was applied (Table 3). The flow-rate was set to 1.0 mL/min, the column temperature to 25 °C, and the injection volume was 200 µL. The DAD wavelength was set at 285 nm for CBZ and 266 nm for SMZ. Instrument control, data acquisition and evaluation were carried out with the ChemStation B.03.01 Software. This procedure was performed for the five standard concentrations used, to determine extraction SPE method recoveries for the five standard concentrations used, for a spiked effluent sample (used for quality assessment), and for the effluent sample.

The identification of the selected pharmaceuticals in the effluent sample was ensured by monitoring CBZ and SMZ retention times under the same analytical conditions for both the sample and the certified analytical standard (CBZ: Sigma-Aldrich 94496, ≥99% purity; SMZ: Sigma-Aldrich S7507, analytical standard grade) solutions prepared in MeOH:deionized water (25:75 *V/V*). Moreover, for a more rigorous peak assignment, the technique of 'spiking' the effluent sample with a CBZ/SMZ standard mixture of 5 µmol/L each was performed. An analytical quality-assurance study was achieved, which included determination of linearity, extraction SPE method recovery for the five standard concentrations, method detection limit (MDL) and method quantitation limit (MQL). Linearity was evaluated through the injection of three replicates of a daily-prepared standard mixture of CBZ and SMZ (from their stock solutions) in MeOH:deionized water (25:75 *V/V*). The calibration curve was constructed using five points (0.6, 1.0, 2.5, 4.0 and 5.0 µmol/L). Analyte recoveries were calculated by comparing the peak areas obtained from HPLC analysis of SPE extracts with those from fortified standard solutions at equivalent concentrations. MDL and MQL were calculated as 3 × Sy/x/*b* and 10 × Sy/x/*b*, respectively, where Sy/x is the standard error of the estimated curve and *b* the slope of the calibration curve.

2. Results

Results showed that all the cell-based assays were accepted (validated), since the CV of negative controls never exceeded 3.2%. The 24-hr H9c2(2-1) toxicity results used to select the

Table 4 – Concentration (% of effluent)-response relationship results presented as $EC_{50,24hr}$ data after the sulforhodamine B assay for the three sample pre-treatments and after constraining the bottom of the curve to zero. Bold indicates valid $EC_{50,24hr}$ results.

	80 mL lyophilised	80 mL SPE	250 mL SPE
$EC_{50,24hr}$ (95% CI)	**1793 (1579–2036)%**	5577 (1965–15,828)%	**4748 (4307–5233)%**
Goodness of fit (r^2)	0.893	0.742	0.973
%FE of EC_{50}	5.8	48	4.5

CI, confidence interval.

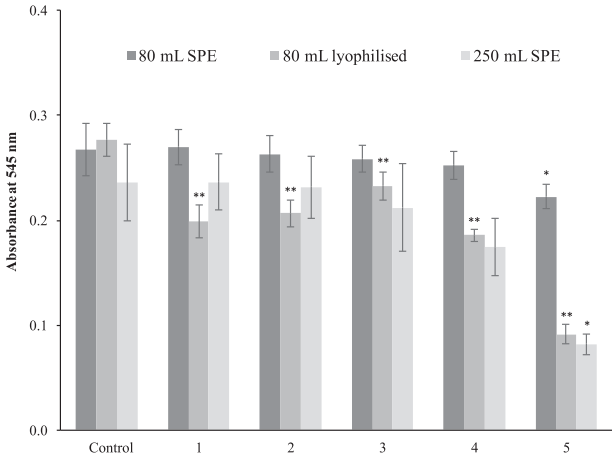

Fig. 1 – Effects of different pre-concentration techniques on H9c2(2-1) cell mass decrease after a 24-hr exposure to a municipal effluent and using the sulforhodamine B assay. Data presented are the mean ± SD of three independent experiments. * P <0.05, ** P <0.001, both denote significant differences from the control. The 1 to 5 x -axis label indicates the five effluent concentrations tested, from the lowest (1) to the highest (5) concentration (see Table 2 of Section 1.3.2).

best concentration pre-treatment (80 mL lyophilised, 80 mL SPE and 250 mL SPE) are presented in Fig. 1 as the mean of absorbance data at 545 nm ± standard deviation (SD) of three independent SRB assays. The five concentrations using the 80 mL lyophilised pre-treatment which were tested showed significant cell mass decrease compared to control, whereas in both SPE pre-treatments only the highest concentration presented a significant difference from the control.

The concentration-response relationship results were presented as $EC_{50,24}h_r$ since the purpose is to relate *in vivo* fish $LC_{50,96hr}$ with *in vitro* $EC_{50,24hr}$ results in subsequent studies. Nevertheless, $EC_{50,24hr}$ were only possible after constraining the bottom of the curve to zero, since data did not define the bottom of the curve (no bottom asymptote), assuming that the cellular response (cell mass) at very high concentrations of the effluent sample is zero. This procedure was only performed to assist in the choice of the best concentration pre-treatment. Not all $EC_{50,24hr}$ results were considered valid, since the one obtained by 80 mL SPE did not meet the target values of goodness of fit >0.850 and %FE <40% (Table 4). Concerning the two valid $EC_{50,24hr}$ results (80 mL lyophilised and 250 mL SPE), to obtain the same result, i.e., to decrease cell mass in 50%, less effluent sample is needed by the lyophilisation method and, in addition, toxicity is 2.7-fold higher. Therefore, lyophilising the sample as a pre-treatment provided the best response from H9c2(2-1) cells, when compared with the SPE concentration method. To confirm that, a 150 mL lyophilised assay was performed, and a valid $EC_{50,24hr}$ of 1329 (1098-1608)% was obtained (r^2=0.961, %FE=8.7) without the need of constraining the bottom of the curve to zero. Moreover, the positive control (2% DMSO) used in the latter assay significantly decreased cell mass (Dunnett's test, P <0.0001).

The performance of the analytical method used for the determination of CBZ and SMZ is presented in Table 5. Linear regression analysis of the concentration ranges produced good fits, with r^2 >0.995. Results also demonstrated that the method achieved satisfactory recoveries, between 80% and 120%.

Table 6 summarizes the results of the physicochemical parameters measured *in situ* and determined in laboratory for the effluent sample. According to the results, nitrates, ammonium and total iron exceeded the emission limit set by the Portuguese legislation (Decree Law 236/98, *Annex XVIII*). The pharmaceuticals CBZ and SMZ were determined in the effluent sample, in concentrations of 0.63 and 0.97 (µg/L), respectively.

3. Discussion

Concerning the best pre-treatment to successfully determine municipal effluent toxicity using cell-based assays, lyophilisation, when compared to SPE, ensured maximum retention of toxic compounds as rat cardiomyoblast H9c2(2-1) cells showed higher sensitivity. It should be highlighted that, when using the SPE technique, some water-soluble components of the sample such as salts and metals could be removed (Snyder and Leusch, 2018), thus eliminating potential toxic compounds. Also, sample pH, the nature of the adsorbent in the cartridge and the elution solvents used affect the efficiency of the extraction, thereby altering toxicity (Togola and Budzinski, 2007). Regarding sample lyophilisation, no manipulation is needed, as this consists of simply removing ice from the sample through the process of sublimation with no alteration of its constituents (even the inherent structure of compounds is maintained, Tao et al., 2013), thus reducing overall sample degradation. Moreover, the lyophilisation process is carried out under vacuum conditions, so there is an absence of air, preventing sample deterioration due to oxidation, and lyophilisation temperature is lower than ambient temperature, resulting in minimal alteration of sample constituents (Barbosa-Cánovas and Vega-Mercado, 1996). Therefore, after a higher volume of the effluent sample was tested to concentrate the sample for clarity of the bottom plateau and to estimate a reliable EC_{50}, and a positive control was performed, sample lyophilisation was considered a more suitable approach for the pre-treatment step of effluent sample concentration to assess realistic toxicity. For municipal effluent samples with prior treatment in a wastewater treatment plant facility, and in order to validate the obtained EC_{50}, a sample of around 150 mL should be collected. It should then be deep-frozen and lyophilised for the H9c2(2-1)-based assays.

As previously concluded for pesticides and pharmaceuticals (Rodrigues et al., 2015, 2019, 2020), the underlying analysis of this earlier study allowed us to determine that sample

Table 5 – performance of the analytical method used for the determination of carbamazepine (CBZ) and sulfamethoxazole (SMZ) in effluent samples.

	Retention time (min)	Linearity		Recovery range (%)	MDL (μg/L)	MQL (μg/L)	250 mL SPE (μg/L)
		r^2	range (μg/L)				
CBZ	16.62	0.999	141.8–1181	83–97	42.5	130.0	311.9
SMZ	12.40	0.999	152–1266	81–95	45.6	139.3	483.8

MDL, method detection limit; MQL, method quantification limit.

Table 6 – Results of the physicochemical parameters measured *in situ* and determined in laboratory for the municipal effluent sample, method quantification limits (MQL), and emission limit values according to the Portuguese legislation (Decree Law 236/98, *Annex XVIII*). Bold indicates no complying parameters. Date of sample collection: May 30, 2018.

	Effluent sample	MQL	Emission limit values
Temperature (°C)	20.4	–	–
pH	7.4	–	6–9
Oxygen (%)	35.9	–	–
Oxygen (mg/L)	3.23	–	–
Electrical conductivity (μS/cm)	1087 (at 25 °C)	–	–
Chemical oxygen demand (mg/L O_2)	69	30	150
Total suspended solids (mg/L)	22	2.0	60
Dissolved silicon (mg/L Si)	3.03 ± 0.032	0.1	–
Dissolved silicon dioxide (mg/L SiO_2)	6.41 ± 0.068	0.1	–
Total phosphorus (mg/L P)	2.0	0.3	10
Dissolved nitrates (mg/L NO_3)	**140**	**1.0**	**50**
Ammonium (mg/L NH_4)	**20**	**0.15**	**10**
Total arsenic (mg/L As)	0.0047	0.001	1.0
Total cadmium (mg/L Cd)	<0.1	0.0001	0.2
Total chromium (mg/L Cr)	0.0072	0.0001	2.0
Total cobalt (mg/L Co)	0.0010	0.0001	–
Total copper (mg/L Cu)	0.0081	0.001	1.0
Total iron (mg/L Fe)	**5.13**	**0.01**	**2.0**
Total lead (mg/L Pb)	0.0015	0.0001	1.0
Total manganese (mg/L Mn)	0.063	0.0001	2.0
Total mercury (mg/L Hg)	0.000012	0.000005	0.05
Total nickel (mg/L Ni)	0.0093	0.0005	2.0
Total zinc (mg/L Zn)	0.020	0.001	–

lyophilisation as a pre-concentration method coupled with the H9c2(2-1) SRB-based assay might be considered a possible alternative *in vitro* model for fish lethal testing to assess the toxicity of municipal effluents. Nevertheless, further efforts should be carried out to confirm this by testing samples using both methods, the H9c2(2-1) SRB-based assay and the fish lethal test, and only then conclude whether this assay is indeed a suitable alternative to fish lethal testing for municipal effluents.

The results of the physicochemical analysis of the municipal effluent tested demonstrated that 20% of the parameters evaluated presented non-compliance with the Portuguese standards. For instance, high dissolved nitrate and ammonium levels, which exceeded the target value of effluent disposal in the water environment, were recorded. Since nitrate is the end result of nitrification, which is a treatment process that begins in ammonium, these high levels can indicate that the wastewater treatment process did not complete nitrification. From an environmental perspective, high dissolved nitrate levels suggest a high potential for eutrophication, which may cause, together with dissolved phosphorus, a dramatic growth of primary producers, as both are essential plant nutrients. Probably due to the addition of ferric chloride for phosphorus removal through chemical precipitation in the Choupal wastewater treatment plant (technical information provided by the Choupal wastewater treatment plant), total iron also exceeded the emission limit value set by Portuguese legislation. For all the other determined parameters which have correspondence with the *Annex XVIII* of Decree Law 236/98 (Portuguese legislation for environmental protection against effluent disposal in the water environment), the gathered results suggest that the effluent will not negatively impact the quality of the receiving environment, the river Mondego.

Several parameters were determined in the present study which are not listed in the *Annex XVIII* of Decree Law 236/98, such as electrical conductivity, dissolved silicon and silicon dioxide, and total cobalt and zinc. When these results were compared with data from other municipal effluent samples, similar values were reported for electrical conductivity: *e.g.*, values between 749 and 1015 μS/cm were seasonally reported for a municipal wastewater treatment plant in South Africa (Odjadjare and Okoh, 2010), and for dissolved silicon: *e.g.*, 4.1 mg/L were measured for the Deer Island Treatment Plant in Boston, USA (Maguire and Fulweiler, 2017). Concerning total cobalt, a maximum concentration value of 0.0025 mg/L was set by Environment Canada (2013) in order to protect aquatic organisms in freshwater environments; the total cobalt concentration found in our effluent sample was 2.5 times lower. With respect to total zinc, the Portuguese environmental quality target value for superficial water was set at 0.5 mg/L (*Annex XXI* of Decree Law 236/98), and thereby, the concentration de-

termined in our effluent sample (0.020 mg/L) is, by far, lower. When comparing effluent levels with target values of surface water, it should be highlighted that the effluent will be subject to a dilution action when it arrives to the receiving waters, and direct comparisons must be carefully undertaken.

In general, higher levels of the pharmaceuticals CBZ and SMZ were determined in the present study when compared with other municipal effluent samples. For instance, worldwide CBZ maximum concentrations between 0.05 (Vietnam) and 2.7 (Germany) μg/L were reported (Afonso-Olivares et al., 2017; Aydin et al., 2017; Bahlmann et al., 2014; Brunsch et al., 2018; Gerrity et al., 2011; Kosma et al., 2014; Metcalfe et al., 2003; Nakada et al., 2017; Nelson et al., 2011; Nguyen et al., 2018; Paíga et al., 2019; Petrie et al., 2017; Rivera-Jaimes et al., 2018; Spongberg and Witter, 2008; Subedi et al., 2017; Yu et al., 2013; Zhang et al., 2018), with a geometric mean value of 0.48 μg/L (N=17). Concerning SMZ, maximum concentrations between 0.11 (Portugal and Slovakia) and 2.0 (Mexico) μg/L were reported (Afonso-Olivares et al., 2017; Batt et al., 2007; Birošová et al., 2014; Gerrity et al., 2011; Kosma et al., 2014; Nelson et al., 2011; Paíga et al., 2019; Petrie et al., 2017; Rivera-Jaimes et al., 2018; Spongberg and Witter, 2008; Subedi et al., 2017; Tran et al., 2016; Zhang et al., 2018), with a geometric mean value of 0.43 μg/L (N=13). In the broad study of Hughes and colleagues, maximum concentrations of 11.6 and 11.9 μg/L were reported for CBZ and SMZ in freshwater ecosystems, respectively (Hughes et al., 2013), which are higher values than the ones quantified in our effluent sample (CBZ: 0.63 μg/L, SMZ: 0.97 μg/L) and those found in literature. Therefore, such pharmaceutical residues require a thorough evaluation in what concerns the risks for aquatic organisms. For instance, the Netherlands' National Institute for Public Health and the Environment proposed the freshwater quality standards of 1600 and 0.5 μg/L for short- and long-term exposure to CBZ, respectively (RIVM, 2014), and the proposed environmental quality standards (EQS) of the Swiss Ecotox centre (https://www.ecotoxcentre.ch) were 2000 and 2.0 μg/L for short- and long-term exposure, respectively. Concerning SMZ, a long-term freshwater PNEC of 8.98 μg/L derived by a probabilistic methodology and by using European data was set by Straub (2016), and the EQS proposed by the Ecotox centre were 2.7 and 0.6 μg/L for short- and long-term exposure, respectively. In the present study, the values determined for CBZ (0.63 μg/L) and SMZ (0.97 μg/L) surpasses the lower protective values of 0.5 and 0.6 μg/L found for long-term exposure, respectively, and even though dilution should be considered, the monitoring of these compounds should be implemented.

Even though some chemical parameters are above the regulatory limits (corresponding to 20% of the analysed parameters mentioned in the Portuguese Decree Law 236/98), and determined CBZ and SMZ concentrations indicate possible long-term adverse effects on the aquatic life, an effective negative effect at the cellular level only occurred after concentrating the effluent sample more than eight times ($EC_{10,24hr}$ = 799 (589–1084)%). Hence, when the biological and physicochemical data were integrated, no potential high adverse effects were expected on the aquatic organisms of the river Mondego by the discharge of this municipal effluent. Nevertheless, as effluent composition is highly variable as a function of time, day of the week and season, further studies using the H9c2(2-1) SRB-based assay should be performed in the same effluent with samples collected at different time points to ascertain temporal variability of effluent toxicity.

4. Conclusion

To conclude, sample lyophilisation as a pre-concentration method coupled with the H9c2(2-1) SRB-based assay was considered suitable for biological effluent toxicity assessment, which is included in the Whole Effluent Assessment regulatory approach, and could also be used by private companies to assess their own discharge quality and possible impacts. Due to its response, the rat cardiomyoblast H9c2(2-1) cell line might be a possible alternative *in vitro* model for fish lethal testing to assess the toxicity of municipal effluents and fully understand its potential toxic effects. The physicochemical status of the sample suggests a high potential for eutrophication, and iron exceeded the permissible level for wastewater discharge possibly due to the addition of ferric chloride for wastewater treatment. In general, the levels of the pharmaceuticals carbamazepine and sulfamethoxazole are higher than those reported for other countries, and both surpassed the aquatic protective values for long-term exposure.

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