The H9c2(2-1) cell-based sulforhodamine B assay is a non-animal alternative to evaluate municipal wastewater quality over time

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Abstract

In the context of alternatives to animal testing, and since wastewater composition is highly variable, depending on time, day of the week and season, the present study validates the potential of the H9c2(2 – 1) cell-based sulforhodamine B (SRB) assay to evaluate the temporal variability of municipal wastewater. The impact of effluent disposal on water quality was also assessed using indicators of possible nutrient enrichment in the receiving water, and by determining ammonium, pH, chemical oxygen demand, total suspended solids and nine metal elements. Since rainwater can enter the municipal wastewater collection system and possibly alter toxicity, precipitation data was considered, as well as the number of new Covid-19 cases per week in the municipality. Moreover, the efficiency of the wastewater treatment process was evaluated as both raw influent and treated effluent samples were analysed. Results revealed that the H9c2(2 – 1) cell-based SRB assay appears to have an enormous potential to evaluate the temporal variability of municipal effluents, to discriminate influent and effluent toxic characteristics, as well as to study Covid-19 progression. We were able to trace the development of this pandemic by relating positive rates (wastewater may also reflect asymptomatic individuals), a crucial metric for understanding the development of a pandemic, with the selected alternative *in vitro* platform. Finally, the gathered results alerted to the impact of phosphates in the aquatic environment, leading us to recommend the inclusion of this parameter in regulatory assessments of wastewater discharges.

1. Introduction

Municipal wastewater samples may represent an important ethical source of information since their composition reflects the excretion of compounds linked to the consumption of pharmaceuticals (Gerrity et al., 2011; Rodrigues et al., 2020; Thiebault et al., 2019) and illicit drugs (Gerrity et al., 2011; Thiebault et al., 2019) by local populations, or the presence of pathogens circulating in a community (Xu et al., 2005). Wastewater analytical results could, therefore, reveal possible health disorders, consumption patterns and social events (Gerrity et al., 2011; Thiebault et al., 2019), and wastewater-based disease information supports the effective surveillance of a disease, possibly preventing outbreaks. For instance, the EU monitors near-real-time data on geographical and temporal trends of drug-taking habits (https://www.emcdda.europa.eu), and a common approach to establish a systematic surveillance of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its variants in wastewaters was recently recommended (Commission Recommendation 472, 2021). Passive exposure to other chemical compounds such as herbicides applied to control weeds in municipal parks and along roadsides (Sutton et al., 2019), as well as meteorological events (Thiebault et al., 2019), can also be reflected in wastewater samples.

Nowadays the world faces increasing pressure on its water resources, and wastewater recycling and reuse are key subjects for rational management and research. The development of reliable methods to assess the biological component of wastewater toxicity is required to evaluate possible hazards to human health and guarantee the quality of treated wastewater (OSPAR Convention, 2000). It is known that wastewater outlet effluents may be major contributors to numerous water pollution problems, with negative consequences to aquatic life and human health (Aristi et al., 2015; Hamdhani et al., 2020). Thus, several *in vitro* cell-based assays have been proposed to characterize the biological component of wastewater toxicity (e.g., Rodrigues et al., 2020a), and to evaluate the efficacy of wastewater treatment and recycling processes (e.g., Shrivastava et al., 2017). Accordingly, we have previously shown that the rat cardiomyoblast H9c2(2 – 1) cell-based sulforhodamine B (SRB) colorimetric assay is suitable to quantitatively estimate the biological component of effluent toxicity (Rodrigues et al., 2020a), and as a promising *in vitro* method to replace fish lethal testing of municipal effluents, both in relative and absolute terms (Rodrigues et al., 2021).

Since wastewater composition is highly variable as a function of time, day of the week and season, and in line with our previous studies, the present work aims to confirm the potentiality of the H9c2(2 – 1) cell-based SRB assay to evaluate the temporal variability of municipal wastewater collected in the centre of Portugal during the 2020-year-period. Since rainwater (storm water) can enter the municipal wastewater collection system and impair the performance of treatment facilities (Hummel et al., 2018), influence wastewater quality parameters (Suchowska-Kisielewicz and Nowogoński, 2021), and contribute to the spread of human pathogens (Cann et al., 2013; Olds et al., 2018), daily rainfall data were also evaluated, as well as Covid-19 reports (number of new cases per week in the municipality covered by the selected wastewater treatment plant). The impact of effluent disposal on water quality was assessed using indicators of possible nutrient enrichment in the receiving water, with the determination of phosphates (PO_4^{3-}), nitrates (NO_3^-) and silicates (Si). Other parameters, as ammonium (NH_4^+), pH, chemical oxygen demand

(COD), total suspended solids (TSS) and nine metal elements, were determined in the samples in order to verify compliance with the specific discharge limits required by the Portuguese legislation for environmental protection against wastewater disposal in the water environment (Decree-law 236/98, 1998, *Annex XVIII*). Finally, the efficiency of the wastewater treatment process was evaluated as both raw influent and treated effluent samples were analysed.

The present study validates the use of the H9c2(2 – 1) cell-based SRB assay for municipal wastewater testing over time, providing helpful information about the potential use of this non-animal assay as an efficient tool, which can offer trend population data to complement other surveillance information so as to influence public health decision-making at the local level. Based on the broad data obtained, this study will also clarify the importance of wastewater-based analysis to estimate the risk for aquatic organisms, as well as the need to develop technologies at wastewater treatment plant level so as to reduce pollutant output.

2. Material And Methods

2.1 Characterization of the wastewater treatment plant

The municipal wastewater treatment plant selected for the present study collects and treats hospital wastewater from an hospital with 1736 beds and 297,654 urgency admissions (data from 2018, https://www.chuc.min-saude.pt), and is also responsible for the treatment of the raw wastewater of 140,796 residents (Portuguese 2021 census). This wastewater treatment plant uses a pre-treatment of the influent consisting of three mechanical cleaning grilles and two desanders, two circular decanters for the primary treatment process, and a secondary treatment composed of four percolating beds with rotary distributors and ventilation channels and two circular secondary decanters.

2.2 Sampling procedures and sample preservation

The wastewater samples were collected monthly (inlet influents) and weekly (outlet effluents) during the year 2020, between Saturdays and Mondays, in a total of 11 influent (in January no sample was collected) and 52 effluent samples. The time of grab sampling is reported in Table S1 (supplementary material). For sample collection, an amber glass container was completely filled (≈2.5 L) and transported to the laboratory (≈20 min), where it was immediately processed or refrigerated at 4 ºC to be processed within 24 hours. Nevertheless, pH and electrical conductivity (µS cm^{-1}) were always measured on arrival at the laboratory, and a well-mixed measured volume of the sample was filtered through a pre-weighed glass microfiber filter (Whatman GF/F 1825047) and placed in an oven until constant weight, to be further re-weighed for TSS determination. Then, for PO_4^{3-}, NO_3^-, Si and NH_4^+ determinations, 500 mL of raw sample were frozen (-18 ºC) in PET containers. For COD determinations, 25 mL of raw sample were acidified to a pH below 2 with H_2SO_4 (Sigma-Aldrich 320501) and preserved at 4 ºC in glass containers until analysis. For metals (arsenic (As), iron (Fe), lead (Pb), cadmium (Cd), chromium (Cr), copper (Cu), manganese (Mn), mercury (Hg) and nickel (Ni)) determination, 120 mL of raw sample were acidified to pH below 2 with 65% HNO_3 (Panreac 213255) and stored in a glass container. The accuracy of the analyses was verified against triplicated blanks using ultra-pure water. The four glass containers used (one for the sample and three for blanks) were previously acid-washed overnight with 65% HNO_3, thoroughly rinsed with ultra-pure water, dried before sample storage, and then preserved at 4 ºC until analysis. Finally, for cell-based assays, 200-mL of filtered (Whatman GF/F 1825047) sample were deep-frozen (-80 ºC) in glass containers for further lyophilisation (Martin Christ Alpha 1–2 Ldplus).

2.3 Chemistry-based assessment

Total suspended solids (mg L^{-1}) determination was assessed by the difference of glass microfiber filter weights (before and after sample filtration), whose mass increase was divided by the wastewater volume filtered. Determinations of PO_4^{3-}, NO_3^-, Si and NH_4^+, as well as COD, were performed by photometric analysis using a Palintest Photometer 7500 after calibration was verified (Palintest standards PT804), and using the photometer Phot 28 method for PO_4^{3-} (mg PO_4^{3-} L^{-1}), the Phot 63 method for NO_3^- (mg NO_3^- L^{-1}), the Phot 31 method for Si (mg Si L^{-1}), the Phot 04 method for NH_4^+ (mg NH_4^+ L^{-1}), and the Phot 81 method for COD (mg O_2 L^{-1}) determinations. The Palintest procedures were all performed following supplier instructions. Metal elemental analysis (except Hg) were determined using an inductively coupled plasma-mass spectrometer (ICP-MS) Thermo X Series. The ICP-MS was equipped with peristaltic pump, Burgener nebulizer and Ni cones. To ionize the sample molecules (sample flow ≈1

mL min^{-1}), the equipment worked at 1,400 W with an Argon flow of 13 L min^{-1} and an auxiliary gas flow of 0.8 L min^{-1}. The calibration curve was made with multi-element standards diluted from certified standards for ICP analysis. Correlation coefficients under 0.999 were discarded and the error associated with each standard never exceeded 10%. The acceptable relative standard deviation between sample duplicates was < 5%. The quantification of Hg was carried out using cold vapour atomic fluorescence spectroscopy (CVAFS). The CVAFS was equipped with a 10.003 PSA cold vapour generator associated with a 10.023 Merlin PSA detector, and 2% of $SnCl_2$ prepared in 10% of HCl was used as reducing agent. Calibration was performed using at least five acidified standards, which were prepared by dilution of a "BDH" $Hg(NO_3)_2$ standard solution of 1.0 g L^{-1}. Confirming the calibration status of the equipment, a standard was analysed between every three samples to check for instrument drift. The acceptable relative standard deviation between sample triplicates was < 5%. The method detection limits and method quantitation limits by metal element were: As (2.0 and 5.0 µg L^{-1}), Fe (10 and 25 µg L^{-1}), Pb (0.1 and 0.25 µg L^{-1}), Cd (0.1 and 0.25 µg L^{-1}), Cr (1.0 and 2.5 µg L^{-1}), Cu (2.0 and 5.0 µg L^{-1}), Mn (0.2 and 0.5 µg L^{-1}), Hg (0.1 and 0.25 µg L^{-1}), and Ni (1.0 and 2.5 µg L^{-1}).

2.4 H9c2(2 − 1)-based assays

H9c2(2 − 1) cells came from the ATCC cell bank (CRL-1446) and were grown in a sterile environment using a humidified atmosphere with 5% of CO_2 at 37 ºC (CO_2 Unitherm, UniEquip). An adherent cell monolayer was cultured in dishes (VWR 734–2321) with filtered (Autofil 1102-RLS) DMEM-high glucose (Sigma-Aldrich D5648) culture medium adjusted to contain 1.8 g L^{-1} of sodium bicarbonate and supplemented with 10% of fetal bovine serum (Gibco 10270-106) and 1% of antibiotic-antimycotic (Gibco 15240-062), at pH 7.3.

Based on the stoichiometric ability of the SRB dye to bind to protein components of cells, the SRB assay was selected to evaluate cell growth inhibition potential of wastewater samples (Vichai and Kirtikara, 2006). To reduce possible cell passage effects, a maximum cell passage number of #20 was used in all the SRB assays performed. For that, H9c2(2 − 1) cells were plated the day prior to the assay at 10^4 cells mL^{-1} density in 48-well plates (Corning 3548). To prepare the exposure solutions, 4.0 mL of cell culture medium was added to each lyophilised sample, and a sonicator (Branson Ultrasonics, 3510E-DTH) was used for 2 min to promote homogenisation. Then, in the highest concentration well, 400 µL of the well medium were removed and replaced with 900 µL of exposure solution, and then serial dilutions were applied, making a test concentration range of 35.2-4,500%. Three replicates prepared from independent cell cultures (true replicates) were maintained for each concentration, and four negative (cells with culture medium alone) and four positive (cells with 2% DMSO prepared in culture medium) controls were considered by replicate. After a 24-h exposure time, cells were washed with phosphate buffer solution, dried, and then fixed at -18 ºC with cold 1% of acetic acid prepared in methanol (Honeywell 34885). The fixative was removed and cells were stained for 60 min at room temperature using SRB solution (prepared in 1% of acetic acid in ultra-pure water) and excess of dye was removed by washing the wells with 1% of acetic acid prepared in ultra-pure water. Protein-bound dye was dissolved under gentle stirring using 10 mM Tris/base (Sigma-Aldrich T1503) at pH 10 and quantified from absorbance measurements (545 nm) using a microplate reader (BioTek Synergy HT).

2.5 Validity criteria and statistical analysis

To ascertain reproducibility and as plate acceptance criteria, the coefficient of variation of the mean (CV, in percentage) was calculated for the negative controls (Iversen et al., 2012). CV was calculated by the equation:

$$CV\ (\%) = (SD/\sqrt{n})/\text{arithmetic mean} \cdot 100$$

where SD is the standard deviation and *n* the number of negative control wells per independent experiment. The acceptance criterion is CV ≤ 20%.

Non-linear regression analysis and curve fitting parameter were performed to calculate EC_{50s} (95% confidence interval). For that, absorbance SRB data were expressed as a fraction of the controls. Then, a four-parameter logistic regression after log-transformation of x-axis values was applied (GraphPad Prism 6 software). To validate the EC_{50s} results, the fitted concentration-response curves should have a r^2 (coefficient of determination) ≥ 0.85, and the percent fitting error (%FE) of EC_{50} must be ≤ 40% (Beck et al., 2017). FE was calculated by the equation:

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

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

After verifying normality (Shapiro-Wilk test) and homogeneity (Levene's test) assumptions, and to statistically detect significant differences between SRB negative and positive controls, the Student's *t*-test (independent groups) was selected, and a significance threshold of 0.05 was considered (STATISTICA 7 software). This test was also used to evaluate the wastewater treatment process by statistically detecting significant differences between influent and effluent monthly data (for PO_4^{3-}, NO_3^-, Si, TSS, Fe, Mn and $EC_{50,24h}$ data sets, logarithmic data transformation was previously performed).

Since both influent and effluent H9c2(2 – 1) $EC_{50,24h}$ data sets do not follow Gaussian distribution, the Spearman nonparametric correlation was selected to evaluate possible monotonic statistical relationships between cell-based data and the 12 variables chosen (STATISTICA 7 software), and the Bonferroni correction was applied to deal with multiple testing by adjusting the significance level (Zar, 2010).

3. Results And Discussion

Supplementary Tables S1 (nutrients, NH_4^+, pH, COD and TSS) and S2 (metal elements) summarize the results of the physicochemical parameters for the wastewater samples. Regarding metals, As, Cd and Hg were always below the quantification limit of the method throughout the year, whereas all the others (Fe, Pb, Cr, Cu, Mn and Ni) occurred in a frequency that varied between 27.3 (Cr) and 100% (Fe and Mn) in the influent samples, and between 17.3 (Cr) and 100% (Fe and Mn) in the effluent samples. The H9c2(2 – 1) cell-based SRB results showed that all the assays were accepted (validated), with the CV of negative controls never exceeding 4.4% (Table 1). Moreover, the positive controls (2% DMSO) always significantly decreased cell mass ($P < 0.05$). The 24-h cell toxicity results were also presented in Table 1. In 9.6% of the cases the effluent $EC_{50,24h}$determination, despite reportable, was not valid because of the low number of more extreme assay concentrations on the lower plateau, i.e., the sample presented low toxicity and, thus, a larger sample volume should have been lyophilised. Therefore, in those cases, a valid concentration-response curve was only possible after constraining the bottom of the curve to zero.

Table 1
Validity data and concentration-response relationship results of H9c2(2 − 1) SRB-based assays from municipal wastewater samples.

Date	Influent				Effluent			
	CV Max (%)	FE (%)	Goodness of fit (r^2)	$EC_{50,24h}$ (95% CI) (%)	CV Max (%)	FE (%)	Goodness of fit (r^2)	$EC_{50,24h}$ (95% CI) (%)
Jan 4	-	-	-	-	1.8	4.7	0.975	2493 (2263–2748)*
Jan 11	-	-	-	-	1.6	7.4	0.987	1514 (1299–1766)
Jan 19	-	-	-	-	4.1	4.9	0.958	2777 (2506–3078)*
Jan 26	-	-	-	-	1.4	23	0.913	1842 (1139–2978)
Feb 2 or 4	1.0	4.7	0.984	365 (330.9-403.3)	1.8	6.3	0.942	2912 (2555–3320)*
Feb 9	-	-	-	-	2.4	22	0.941	1172 (733.7–1871)
Feb 16	-	-	-	-	2.8	11	0.942	1888 (1503–2373)
Feb 23	-	-	-	-	2.6	22	0.887	1810 (1145–2861)
March 1	2.4	4.9	0.986	630 (569.5-697.4)	1.9	18	0.910	1057 (731.6–1527)
March 8	-	-	-	-	2.2	12	0.937	941 (739.3–1198)
March 14	-	-	-	-	2.6	7.3	0.959	2161 (1858–2514)*
March 22	-	-	-	-	3.2	4.2	0.983	597 (547.8-651.5)
March 29	-	-	-	-	1.5	12	0.962	968 (747–1255)
April 6	2.0	22	0.921	775 (486.0-1235)	2.3	5.4	0.977	1279 (1142–1433)
April 13	-	-	-	-	2.9	8.1	0.967	760 (641.7-899.3)
April 19	-	-	-	-	2.7	10	0.926	919 (741.3–1138)
April 27	-	-	-	-	2.2	3.4	0.990	528 (491.6–567.0)
May 4	3.5	7.2	0.967	400 (344.4-464.2)	3.3	9.9	0.962	914 (743.8–1123)
May 11	-	-	-	-	1.5	10	0.926	644 (521.1-794.7)

CV, coefficient of variation of the mean; FE, fitting error; CI, confidence interval
*after constraining the bottom of the curve to zero

Date	Influent				Effluent			
	CV Max (%)	FE (%)	Goodness of fit (r^2)	$EC_{50,24h}$ (95% CI) (%)	CV Max (%)	FE (%)	Goodness of fit (r^2)	$EC_{50,24h}$ (95% CI) (%)
May 18	-	-	-	-	1.4	11	0.929	480 (383.7-599.2)
May 25	-	-	-	-	1.6	7.3	0.971	493 (423.6-573.9)
May 30	-	-	-	-	1.7	11	0.973	801 (639.2–1003)
June 8	1.1	6.3	0.975	476 (417.5-542.5)	2.1	12	0.921	451 (351.7-578.1)
June 15	-	-	-	-	2.1	9.5	0.970	995 (816.7–1212)
June 22	-	-	-	-	4.4	11	0.937	996 (785.8–1263)
June 29	-	-	-	-	3.6	8.6	0.966	1116 (932.3–1335)
July 6	1.4	6.4	0.971	707 (619.1-807.3)	3.7	14	0.941	1098 (825.8–1460)
July 13	-	-	-	-	2.7	5.2	0.939	3681 (3305–4101)*
July 20	-	-	-	-	1.9	8.6	0.983	1021 (852.7–1221)
July 27	-	-	-	-	2.7	7.8	0.964	1910 (1623–2248)
August 3	0.9	11	0.952	519 (411.3-655.5)	0.83	32	0.946	1178 (602.5–2303)
August 8	-	-	-	-	2.8	15	0.893	851 (621.9–1165)
August 17	-	-	-	-	2.2	27	0.953	1364 (771.0-2412)
August 24	-	-	-	-	1.7	12	0.970	1055 (823.6–1351)
August 31	-	-	-	-	1.0	23	0.981	1422 (872.3–2320)
September 5	3.6	14	0.886	419 (314.5-558.5)	2.7	6.5	0.982	638 (556.8-731.3)
September 14	-	-	-	-	2.8	15	0.971	818 (594.4–1124)
September 21	-	-	-	-	1.9	9.4	0.931	1677 (1379–2039)
September 28	-	-	-	-	1.0	13	0.956	1030 (786.4–1349)

CV, coefficient of variation of the mean; FE, fitting error; CI, confidence interval
*after constraining the bottom of the curve to zero

Date	Influent				Effluent			
	CV Max (%)	FE (%)	Goodness of fit (r^2)	$EC_{50,24h}$ (95% CI) (%)	CV Max (%)	FE (%)	Goodness of fit (r^2)	$EC_{50,24h}$ (95% CI) (%)
October 3	2.5	11	0.954	341 (268.2-433.1)	3.9	12	0.981	908 (702.4–1174)
October 12	-	-	-	-	1.6	4.2	0.986	962 (880.8–1050)
October 19	-	-	-	-	1.3	5.1	0.984	901 (809.8–1001)
October 26	-	-	-	-	1.2	16	0.910	1456 (1046–2027)
November 2	2.1	5.5	0.978	354 (315.4-396.6)	2.2	10	0.956	932 (753.8–1151)
November 9	-	-	-	-	1.9	8.3	0.980	832 (699.8-987.9)
November 16	-	-	-	-	1.9	6.0	0.972	652 (575.3-738.1)
November 23	-	-	-	-	1.8	9.4	0.983	800 (657.8-972.5)
November 30	-	-	-	-	0.8	5.6	0.983	479 (426.7-538.3)
December 7	2.2	9.9	0.943	1668 (1356–2053)	1.8	11	0.955	813 (640.4–1032)
December 14	-	-	-	-	1.9	7.9	0.973	979 (829.2–1155)
December 21	-	-	-	-	1.6	10	0.971	717 (585.9-876.8)
December 26	-	-	-	-	3.2	8.2	0.967	498 (419.3-591.4)

CV, coefficient of variation of the mean; FE, fitting error; CI, confidence interval
*after constraining the bottom of the curve to zero

In order of characterise the ability of the H9c2(2 − 1) cell-based SRB assay to assess the temporal variability of municipal wastewater, confirming whether cell-based results reveal wastewater composition, correlation coefficients between H9c2(2 − 1) $EC_{50,24h}$ data and PO_4^{3-}, NO_3^-, Si, NH_4^+, COD, TSS, Fe and Mn were calculated. In line with the precautionary approach, correlations were only possible for COD (for influence data), as the missing value of November was completed with the maximum value determined during the year, as well as for Pb and Ni (for both influent and effluent data-sets) as gaps (when values were below MQL) were completed with a value immediately lower of MQLs: 0.24 µg L^{-1} for Pb and 2.4 µg L^{-1} for Ni. Seeing that there is a mixture of domestic and rain water in the collector of the old town covered by the selected wastewater treatment plant (technical information provided by the wastewater treatment plant), daily rainfall data (mm) provided by the Portuguese Institute for Sea and Atmosphere was also used to assess whether this meteorological event disrupts the wastewater treatment system, while Covid-19 data was used to assess whether the presence of SARS-CoV-2 viral particles would affect H9c2(2 − 1) cells. The freeze-drying technique, used in the present study to concentrate wastewater samples prior to cell-based assays, has the potential to preserve virus infectivity for several years (Adams, 2007). It is also the preferred method for stabilizing live attenuated virus vaccines for long-term preservation and worldwide distribution (reviewed by Hansen et al. (2015)), and to preserve viruses' collections (Baronti et al., 2021). Possibly due to the low number of data pairs (N = 11), correlations revealed that for influent data

no significant relationships were observed between H9c2(2 – 1) results ($EC_{50,24h}$) and the selected variables (Fig. S1, supplementary material), whereas H9c2(2 – 1) results covariated negatively with four variables (PO_4^{3-}, COD, TSS and Covid-19) and positively with one variable (Mn) for effluent data (Table 2, or see the complete Spearman correlation matrix on Fig. S2 of supplementary material). This means that toxicity $EC_{50,24h}$ values decrease (indicating higher toxicity of the wastewater) whenever PO_4^{3-}, COD and TSS values increase, as well as whenever Covid-19 positive case reports increase; and that toxicity $EC_{50,24h}$ values decrease when Mn decreases. Note that the contrary is also true. The correlation results suggest that in aquatic environments PO_4^{3-} is a major contributor to biological impairment, and should, therefore, be included in the list of variables (as COD and TSS are) with a specific discharge limit for environmental protection against wastewater disposal in the water environment. Moreover, H9c2(2 – 1) cells appear to be a promising platform for modelling Covid-19 outbreaks using effluent samples, and possibly for biochemical studies of SARS-CoV-2 replication. The cellular receptor of SARS coronaviruses, the angiotensin converting enzyme 2 (ACE-2) (Li et al., 2003), is known to be expressed on cardiomyocytes (Gallagher et al., 2008), and thus, in addition to respiratory illness, SARS-CoV-2 might initiate a cascade of deleterious events associated with cardiac injury, arrhythmia and cardiac arrest (reviewed by e.g., Arévalos et al., 2021, Bugert and Kwiat et al., 2021). Regarding Mn, the determined levels were in line with its normal range in drinking water (recommended safety limit is 50 μg L^{-1}, Decree-law 152, 2017), and thus a positive significant relationship was observed as it is known that this transition metal is an important cofactor nutrient and a structural component of many proteins, playing a vital role in the cellular metabolism. The same result would be expected for Fe, Cr, Cu and Ni, as they all serve important cellular roles (Andreini et al., 2008). However, the failure of a significant correlation with Fe is possibly because this metal element presented values above the Portuguese discharge limit in 29% of the sampled weeks (Table S2, supplementary material). For Cr and Cu no correlation analysis was performed due to their low occurrence frequency. The very low concentrations determined for Ni: maximum concentration of 7.4 μg L^{-1}, which is even lower than the recommended safety limit of 20 μg L^{-1} for drinking water (Decree-law 152, 2017), could be a reason for the failure of a significant correlation. Correlation analysis also reveals that the effluent levels of NO_3^-, Si and NH_4^+ throughout the year do not impact the biological cell model selected for the present study, and neither does precipitation. The H9c2(2 – 1) cell-based SRB assay provided thus an estimate of the overall toxic burden of a mixture of pollutants present in municipal effluents over time. Gathered results corroborate previous findings that demonstrate the high sensitivity of H9c2(2 – 1) cells to environmental pollutants as pesticides (Rodrigues et al., 2015, 2019), pharmaceuticals (Bains et al., 2013; Rodrigues et al., 2020b), industrial chemicals (Han et al., 2017), charged polymers and heavy metals (Mohammad and Arfin, 2013), as well as toxins (Neves et al., 2020; Varela et al., 2020).

Table 2
Summary of the Spearman correlation results applied to H9c2(2 – 1)-based SRB results ($EC_{50,24h}$) and abiotic variables of effluent samples collected weekly throughout 2020. Bold indicates significance (significance level at 0.0042).

H9c2(2 – 1)-based SRB results ***vs***												
	PO_4^{3-}	NO_3^-	Si	NH_4^+	COD	TSS	Fe	Pb	Mn	Ni	pp	Covid-19
r	-0.526	-0.143	-0.165	-0.066	-0.610	-0.428	-0.034	0.179	0.485	0.071	-0.097	-0.591
P	**< 0.0001**	0.3121	0.2440	0.6388	**< 0.0001**	**< 0.0042**	0.8105	0.2053	**< 0.0042**	0.6154	0.4943	**< 0.0001**
N	52	52	52	52	52	52	52	52	52	52	52	52

PO_4^{3-}, phosphates; NO_3^-, nitrates; Si, silicates; NH_4^+, ammonium; COD, chemical oxygen demand; TSS, total suspended solids; Fe, iron; Pb, lead; Mn, manganese; Ni, nickel; pp, precipitation.

The impact of effluent disposal on water quality was also studied, and according to the results, several effluent data were non-compliant with the Portuguese standards, namely NO_3^- (in 26.9% of the samples), NH_4^+ (88.5%), COD (9.6%), TSS (3.9%) and Fe (28.9%) (Tables S1 and S2, supplementary material). The high values of Fe are probably due to the fact that this wastewater treatment plant uses ferric chloride as orthophosphate precipitation agent (technical information provided by the wastewater

treatment plant). Since effluent NH_4^+ presented high levels during almost the whole year (non-compliance frequency of 88.5%), the biological nitrification process implemented in the wastewater treatment plant seems to require some kind of upgrade (e.g., properly sized lagoon aeration system), or a better control process (e.g., better monitoring of dissolved oxygen, biochemical oxygen demand, pH or temperature levels). To a smaller extent, denitrification (that converts NO_3^- in nitrogen gas) seems to also need improvement.

The H9c2(2 − 1) cell-based SRB assay was effective to discriminate influent and effluent toxic characteristics, as a significant difference was obtained by testing the two $EC_{50,24h}$ data sets ($P < 0.01$), with effluents being 83.1% (mean value) less toxic to H9c2(2 − 1) cells than influents, thus demonstrating the success of this assay to evaluate the toxicity reduction of the wastewater treatment process. By comparing mean values of influent and effluent data, the wastewater treatment process allowed the expected increase of NO_3^-due to nitrification in a percentage increase of 227.4%, and the effective reduction of PO_4^{3-}, NH_4^+, COD and TSS in a percentage decrease of 77.1, 54.5, 48.4 and 84.5%, respectively (all *P* values < 0,001). Both Si and pH remained unchanged (Si *P* value = 0,828, and pH *P* value = 0.316). Except for Fe and Mn, with an occurrence frequency of 100%, and for Ni which increased frequency, the occurrence frequency between influent and effluent decreased for Pb, Cr and Cu (see Table S2 of the supplementary material). When correspondent influent and effluent samples were compared, which was only possible when concentrations were above the MQL in both determinations, the results showed that Fe, Cu, Pb and Mn presented a significant difference (all *P* values ≤0.05), with an effective decrease of Cu (68% decrease) and Pb (63% decrease) levels, and an increase of Fe (408% increase) and Mn (40% increase) levels, while Ni remained unchanged (*P* value = 0.965). Statistical analysis was not possible for Cr as only one correspondence was found. The H9c2(2 − 1) cell-based SRB assay is thus a suitable bioanalytical tool for detection of non-specific toxicity, and might be routinely applied for water quality monitoring and for surveillance of the efficacy of treatment processes.

4. Conclusion

Based on the results of the present study, we concluded that the H9c2(2 − 1) cell-based SRB assay has an enormous potential to evaluate the temporal variability of municipal effluents, to discriminate influent and effluent toxic characteristics, as well as to study Covid-19 progression. Accordingly, this *in vitro* platform may present itself as a valuable alternative for effluent toxicity assessment in the context of animal alternatives, as well as a bioanalytical tool for water quality monitoring and for surveillance of the efficacy of treatment processes. Finally, the gathered results also alert to the impact of phosphates in a biological system, leading us to recommend the inclusion of this parameter in regulatory assessments of wastewater discharges.

Declarations

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Statements and declarations

Ethical approval

The study does not require ethical approval.

Consent to participate

Informed consent was obtained from the wastewater treatment plant selected for sample collection.

Consent to publish

Authors consent the publication of the study in *Environmental Science and Pollution Research*. Authors warrant that the study has not been published before in any form. Authors transfer to the Publisher the copyright of the study.

Authors contributions

Project administration: ET Rodrigues; Conceptualisation: ET Rodrigues; Supervision: PJ Oliveira; Investigation: ET Rodrigues; Writing- original draft: ET Rodrigues; Writing- review: MA Pardal, PJ Oliveira, E Pereira; Methodology: PJ Oliveira, E Pereira; Funding acquisition: MA Pardal, PJ Oliveira, E Pereira; Formal analysis: ET Rodrigues; Resources: MA Pardal, PJ Oliveira, E Pereira.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and materials

The raw data are available as supplementary material.

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