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Effects of gamma radiation on cork wastewater: Antioxidant activity and toxicity

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HIGHLIGHTS

- Real cork boiling wastewater as case study.
- Gamma radiation was studied as advanced oxidation process.
- Evaluation of the antioxidant activity of cork wastewater after gamma radiation.
- Toxicity assessment performed to study the effects of gamma radiation.

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ABSTRACT

A comprehensive assessment of the toxicity and antioxidant activity of cork boiling wastewater and the effects of gamma radiation on these parameters was performed. Antioxidant activity was evaluated using different methodologies as DPPH radical scavenging activity, reducing power and inhibition of β-carotene bleaching. The results have shown that gamma radiation can induce an increase on the antioxidant activity of cork boiling wastewater. Toxicity tests were performed to access the potential added value of the irradiated wastewaters and/or minimization of the impact for discharge in the environment. Two different methods for toxicity evaluation were followed, bacterial growth inhibition test and cytotoxicity assay, in order to predict the behavior of different cells (prokaryotic and eukaryotic) in the presence of cork wastewater. Non-treated cork boiling wastewater seemed to be non-toxic for prokaryotic cells (*Pseudomonas fluorescens* and *Bacillus subtilis*) but toxic for eukaryotic cells (A549 human cells and RAW264.7 mouse cells). The gamma radiation treatment at doses of 100 kGy appeared to increase the toxicity of cork compounds for all tested cells, which could be related to a toxic effect of radiolytic products of cork compounds in the wastewaters.

1. Introduction

Cork industry is one of the most important industries in Portugal. Cork is the bark of the oak tree which is periodically extracted in 9–12 years in order to produce cork with desirable properties for industrial processing. This raw material is used in a large variety of products ranging from engine gaskets to wine stoppers (Mazzoleni et al., 2005).

Cork processing includes the cork planks immersion in boiling water during 1 h to improve their chemical characteristics, such as elasticity and homogeneity and to make it flat. Normally, the same water is used for several boiling cycles which concentrates it in persistent compounds. The resulting effluent is an aqueous and complex dark liquor with high concentration of cork extracts such

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as phenolic acids, tannins (Minhalma and Pinho, 2001) and 2,4,6-trichloroanisol which are difficult to degrade by conventional treatments. The wastewater produced during this stage represents the main source of wastes (approximately 1500 L per ton of cork). It has low pH and contains high contents of Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD). The nature and the concentration of phenolic compounds make this water a toxic and recalcitrant effluent (Dias-Machado et al., 2006). Based on this fact, this effluent might be a risk for the ecosystem, requiring previous treatment before discharge.

In the literature, studies have been published in the last years about the toxicity of some components of cork wastewater like phenolic compounds mixtures using microcalorimetric method (Chen et al., 2010) and also using *Vibrio fischeri*, *Daphnia magna* and *Lemna minor* as test organisms to evaluate acute and chronic aquatic toxicity (Gomes et al., 2013; Mendonça et al., 2007). Although cork wastewater was classified as ecotoxic for some tested species, the biological effect of real cork wastewater is still unclear, since it is considered that the evaluation of toxicity should be done with organisms that have the ability to persist in a wide range of environments, including soils and surface of plants.

Taking into account this problematic and the inefficiency of the conventional treatments to eliminate the recalcitrant compounds, the use of advanced oxidation processes (AOPs) has become widespread in the world. Several processes for cork boiling wastewater treatment have been reported, such as ozonation (Acero and Benitez, 2004; Gomes et al., 2013; Torres-Socías et al., 2013), Fenton's reagent (Dias-Machado et al., 2006; Guedes et al., 2003) and membrane separation (Benítez et al., 2008; Oliveira et al., 2009; Teixeira et al., 2009). Silva et al. (2004) used the combination of H_2O_2, TiO_2, Fe^{2+} and UV radiation to treat cork cooking wastewater being the photo-Fenton process the more efficient. More recently, Torres-Socías et al. (2013) studied the remediation of cork boiling wastewater using solar photo-Fenton and ozone (alone or in combination with H_2O_2) with different flocculants as physico-chemical pre-treatment. All of these processes proved to be effective for COD removal and biodegradability enhancement, although the use of chemicals increase the pollutant load of wastewaters and can form other species that could interfere on the degradation of the desired compounds (Moon et al., 2011). In a recent study, the efficiencies and equivalent costs of advanced oxidation processes (AOPs) such as ionizing radiation radiolysis, photocatalysis, photolysis and ozonolysis were investigated for the treatment of a simulated textile dye wastewater (Guin et al., 2014). Among these AOPs, the most effective in the mineralization of the effluent at the lowest cost was the ionizing radiation treatment, with a total cost/m^3 of wastewater of 175€. The ionizing radiation is being considered an emergent technology for wastewater treatments (Guin et al., 2014). For example, the use of gamma radiation has been studied as a promising technology with the capacity to reduce the impact of chemical and biological pollution of effluents in the environment (Cabo Verde et al., 2015; Melo et al., 2008). In addition, gamma radiation has been also used to reduce the toxicity and mutagenicity of other industrial effluents. Namely, Jo et al. (2006) observed that gamma radiation (absorbed dose of 20 kGy) decreased the toxicity of the textile raw wastewater. Iqbal et al. (2015) studied the effect of different absorbed doses of gamma radiation on the cytotoxicity and mutagenicity of an effluent of a textile industry. In fact, these authors observed that gamma radiation is a feasible technology for detoxification of pollutants and for the degradation of toxic agents. However, Park et al. (2008) suggested that the use of gamma rays seems to be not effective in the reduction of toxicity of wastewater from a rubber products factory and other toxicants than the destroyed organic compounds could formed. In practice, radiation based pilot sludge treatment plants have been established in New Mexico, USA (gamma radiation); Weldel, Germany (e-beam); Verginia Key, USA (e-beam); Takasaki, Japan (e-beam); Sao Paulo, Brazil (e-beam); Tucuman, Argentina (Gamma); Daejeon, Korea (e-beam) (Cooper et al., 1998; Guin et al., 2014). Furthermore, radiation based commercial sludge treatment plants were also established in Vadodara, India and Munich, Germany (Cooper et al., 1998; Guin et al., 2014). A pilot plant for treating 1000 m3/day of dyeing wastewater with e-beam has been constructed and operated since 1998 in Daegu, Korea in conjunction with the biological treatment facility (Han et al., 2012; Maruthi et al., 2011).

Concerning the cork industry, there are few feasibility studies about gamma radiation in the cork boiling wastewater treatment (Lima et al., 2016; Madureira et al., 2013). The authors observed that this technology could bring added-value to this effluent by increasing the antioxidant activity, while decreasing the organic matter (Madureira et al., 2013). The phenolic compounds present in cork waters are known for their high antioxidant activity (Benitez et al., 2003) and their recovery could represent a potential way to valorise the wastewater from cork industry. On the other hand, our previous studies also indicated a negative effect of radiolytic by-products of cork wastewater model solution on the growth of a microbial community, conducting to the hypothesis that irradiated sample toxicity could increase due to the generated intermediates (Lima et al., 2016). These studies raised some issues to investigate, and further studies are needed to elucidate the effects of gamma radiation on the treatment and potential valorization of cork wastewater.

The aim of this work was to study the antioxidant activity and toxicity of cork cooking water and the influence of gamma radiation treatment on these parameters. Two different methods for toxicity evaluation were applied in order to predict the behavior of different cells (procaryotic and eukaryotic). *Pseudomonas fluorescens* and *Bacillus subtilis* were used in the growth inhibition test as previously reported by Paran et al. (1990). These microorganisms were different from the ones usually used to assess the toxicology of wastewaters and especially from cork industry (Mendonça et al., 2007). On the other hand, two different eukaryotic mammalian cell lines, human and mouse, were used to compare the potential cytotoxicity effects of cork wastewater compounds in different living beings (Ma et al., 2014).

2. Materials and methods

2.1. Reagents, cells and bacterial strains

The cork boiling wastewater samples (5 L) were collected from cork planks immersion boiling tanks at *AMORIM Industrial Solutions*, a production and transformation cork industry located in Coruche, Portugal.

For chemical characterization: gallic acid and Folin-Ciocalteau reagent were obtained from Sigma (St. Louis, MO, USA). Sodium carbonate and sulphuric acid (95–97%) were from Merck (Kenilworth, NJ, USA). Ethanol PA was acquired from Panreac Química SA (Barcelona, Spain) and potassium hydrogen phthalate from Fisher Scientific (Waltham, MA, USA). Ferrous ammonium sulfate (FAS) was acquired from Carlo Erba (Val de Reuil, France) and potassium dichromate from JMGS (Odivelas, Portugal). Mercury sulfate and silver sulfate were purchased from VWR (Radnor, PA, USA).

For antioxidant activiy analysis: 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Standards trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and gallic acid were from Sigma (St. Louis, MO, USA). Methanol and all other chemicals were of analytical grade and obtained from common sources.

For toxicity assay: the bacterial strains used were *Pseudomonas fluorescens* (ATCC® 13525™) and *Bacillus subtilis* (ATCC 6633). Tryptone Soya Broth (TSB) was obtained from Oxoid (Basingstoke, UK).

For cytotoxicity assay: A549 human lung alveolar epithelial cells were obtained from ATCC (ATCC® CCL-185™). Raw 264.7 mouse macrophages were obtained from ATCC (ATCC® TIB-71™). Dulbecco's Modified Eagle's Medium (DMEM), heat inactivated fetal bovine serum (FBS), penicillin-streptomicin, HEPES buffer, non-essential aminoacids, L-glutamine, sodium bicarbonate and sodium pyruvate were obtained from Gibco, Thermo Scientific (Waltham, MA, USA). The Cell Proliferation Reagent WST-1 was obtained from Roche (Indianapolis, IN, USA).

Deionized water produced by a Milli-Q water purification system (Merck Millipore, USA) was used.

2.2. Irradiation experiments

Irradiation experiments were carried out in a Co-60 experimental chamber (model Precisa 22, Graviner Lda, UK, 1971, with four cobalt-60 sources with a total activity of 140 Tbq, 3.77 kCi, May 2015) located at Instalação de Radiações IonizanteS (IRIS) from Centro de Ciências e Tecnologias Nucleares (C^2TN) of IST, Universidade de Lisboa. All irradiations were conducted at a dose rate of 1.6 kGy h^{-1}. The cork wastewater samples in glass bottles (200 mL, three replicas) were irradiated at 20, 50 and 100 kGy, in an automatic rotation system to guarantee dose uniformity. The absorbed doses were measured by routine dosimeters (Whittaker and Watts, 2001). The local dose rate had been previously determined by Fricke method (ASTM, 1992).

2.3. Physico-chemical characterization of cork wastewater samples

The pH was measured by potentiometer (Radiometer, model PHM210) previously calibrated with buffered solutions. Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD) and Total Suspended Solids (TSS) were determined according to the Standard Methods for the Examination of Water and Wastewater (APHA, 1999). Total Phenolic content (TP) was quantified by Folin-Ciocalteau method (Singleton et al., 1998) calibrated with gallic acid. All the assays were made in triplicate.

2.4. Antioxidant acitivy of cork wastewater samples

The antioxidant activity was evaluated by *in vitro* assays based on different mechanisms of action: DPPH radical scavenging activity, reducing power and inhibition of β-carotene bleaching. For these assays, the non-irradiated and irradiated samples were liophilized during 60 h.

2.4.1. DPPH radical scavenging activity

This methodology was performed using an ELX800 Microplate Reader (Bio-Tek). The reaction mixture in each one of the 96-wells consisted in one different concentration of the wastewater samples (30 μL) and methanolic solution (270 μL) containing DPPH radicals (6×10^{-5} mol L^{-1}). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: $\%\,RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract had been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The assay was made in triplicate.

2.4.2. Reducing power

The methodology was performed using the Microplate Reader described above. Different concentrations of wastewater samples (0.5 mL) were mixed with sodium phosphate buffer (200 mmol L^{-1}, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). For each concentration, the mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48-wells plates along with 0.8 mL of deionized water and 0.16 mL of ferric chloride (0.1% w/v). The absorbance was measured at 690 nm. The assay was made in duplicate.

2.4.3. β-Carotene bleaching

β-Carotene (2 mg) was dissolved in chloroform (10 mL) and 2 mL of this solution was pipetted into a round-bottom flask. After the chloroform was removed at 40 °C under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of wastewater samples (0.2 mL). The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm β-Carotene bleaching inhibition was calculated using the following equation: (Absorbance after 2 h of assay/Initial absorbance) × 100. The assay was performed in triplicate.

2.5. Toxicity of cork wastewater samples

The samples of cork wastewater were sterilized by filtration with a 0.2 μm filter and different dilutions were prepared (100%, 50%, 10% and 1% of cork wastewater) in order to study the effect of the concentration in the procaryotic and eukaryotic cellular growth. Standard reference toxicity tests were conducted with potassium dichromate ($K_2Cr_2O_7$) in both tests.

2.5.1. Bacteria growth inhibition assay

This test is based on the evaluation of the effects of the cork wastewater samples on the growth rate of a growing bacterial culture in Tryptic Soy Broth (TSB) under defined conditions of temperature and time (Paran et al., 1990; Paz et al., 2006). The purpose of this test was to verify: a) if the compounds present in cork wastewater are toxic for the growth (inhibitory or bacteriostatic) of *Pseudomonas fluorescens* (Gram-negative bacteria) and Bacillus subtilis (Gram-positive bacteria); b) the effect of gamma radiation in the toxicity of these samples.

For this experiment, saturated cultures (2 mL) of *P. fluorescens* and *B. subtilis* were inoculated into sterile TSB (23 mL) until an $OD_{620nm} = 0.2$ (logarithmic growth phase). The cork wastewater samples (100 μL) described above were inoculated with the bacteria culture (10^3 and 10^2 CFU mL^{-1}) in 96-wells plates. The plates were incubated at 30 °C during 12 h for *B. subtilis* and 24 h for *P. fluorescens*. The optical density of samples and controls were measured at 620 nm before incubation (Time 0) and after 12 h and/or 24 h of incubation using an EZ Read 800 Microplate Reader (Biochrom, Cambridge, UK). The assays were made in triplicate in three independent experiments with three controls: (i) untreated microorganisms in culture medium; (ii) samples in culture medium without microorganisms; and (iii) culture medium only.

2.5.2. Cytotoxicity assay

A549 and RAW264.7 cells were maintained at 37 °C and 5% CO_2, in Dulbecco's modified Eagle medium (DMEM) supplemented with L-glutamine (1 mmol L^{-1}), 10% Fetal Bovine Serum (FBS; Heat inactivated), penicillin (1 U mL^{-1}), streptomycin (100 μg mL^{-1}),

sodium pyruvate (1 mmol L^{-1}), sodium pyruvate (0.1 mmol L-1), non-essential amino acids (0.1 mmol L^{-1}) and HEPES buffer (1 mmol L^{-1}).

Cell viability was measured using the WST-1 cell proliferation assay based on quantification of mitochondrial activity as an indicator of cytotoxicity (Ma et al., 2014). Twenty-four hours before the challenging assay, A549 and Raw264.7 cells (1×10^5 cells mL^{-1}) were seeded into 96-well plates. In the following day, cellular monolayers were washed two times with PBS and fresh 2× concentrated DMEM (100 μL) and cork wastewater samples (100 μL) were added to every well. The cells were incubated with the cork wastewater (non-irradiated and irradiated) samples for 24 h, at 37 °C and 5% CO_2. In the next day, fresh DMEM (100 μL) was added. WST-1 reagent was added to each well at 1/10 volume of the medium. The absorbance was quantified after incubating at 37 °C for 6 h by an EZ Read 800 Microplate Reader (Biochrom, Cambridge, UK) at 450 nm with a reference wavelength of 620 nm. For both cell lines the assays were made in duplicate in two independent experiments with three controls: (i) untreated cells in culture medium; (ii) samples in culture medium without cells; and (iii) culture medium only.

2.6. Data analysis

Origin software version 7.5 (OriginLab Corporation, Northampton, USA) was used for data analysis. Confidence intervals for means values were estimated considering a significance level of $p < 0.05$ and the number of replicates for each assay. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with $\alpha = 0.05$.

3. Results and discussion

3.1. Physico-chemical characterization of cork wastewater samples

The physico-chemical characterization of non-irradiated and irradiated cork wastewater samples is presented in Table 1.

The main characteristics of this wastewater were the low pH, the high value of COD and the low biodegradability, which can be explained by the presence of non-biodegradable compounds. Similar values were reported before by Dias-Machado et al. (2006), with BOD_5 and COD values ranging from 490 to 735 mgO_2 L^{-1} and 2300–4600 mgO_2 L^{-1}, respectively. Concerning Total Phenolics, the observed results are in agreement with those reported by other authors (Dias-Machado et al., 2006; Gomes et al., 2013; Lima et al., 2016) indicating a high concentration of phenolics in this type of wastewaters.

High gamma radiation doses, 20 kGy up to 100 kGy, were applied to cork wastewater samples, based on previous studies which indicated such dose range to be able to degrade the recalcitrant phenolic compounds present in cork wastewater (Lima et al., 2016; Madureira et al., 2013; Melo et al., 2011). Other authors also reported degradation of persistent organic matter by ionizing radiation using high radiation doses up to 75 kGy (Lim et al., 2016).

Regarding the effects of irradiation on physicochemical parameters of cork wastewater, it was verified a reduction of BOD and Total Suspendid Solids, that was significative for both parameters after irradiation of cork wastewater at 100 kGy. These effects could represent a decrease of organic matter content and an increase of chemical species soluble in water. The variations of COD with radiation dose were not significative, indicating no effect of irradiation on the amount of oxidized organic matter. Table 1 shows a reduction of BOD/COD ratios to values lower than 0.1 for cork wastewater treated with gamma radiation, which reflects the non-biodegradable character of irradiated effluent. An increasing trend of Total Phenolics content with irradiation was also observed, although only significant for TP parameter in cork wastewater irradiated at 100 kGy. This effect could be attributed to the radiolytic degradation of large phenolic molecules into small ones. These results suggested that gamma radiation is a potential technology for cork wastewater treatment since the BOD and TSS values are greatly reduced (87% and 45%, respectively) at higher doses (100 kGy). Moreover, the increase of 21% of TP content in cork wastewater irradiated at 100 kGy stressed the potential valorization of irradiated effluent, due to the documented correlation between TP content and antioxidant activity (Benitez et al., 2003).

3.2. Antioxidant activity of cork wastewater samples

The antioxidant activity of cork wastewater samples and the effect of gamma radiation treatment on this parameter are presented in Table 2.

The antioxidant activity was expressed as EC_{50} values (Mean ± SD), what means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC_{50} values: 41 μg/mL (reducing power), 42 μg/mL (DPPH scavenging activity) and 18 μg/mL (β-carotene bleaching inhibition).

The irradiated samples tend to have a higher antioxidant activity (lower EC_{50} values) than the non-irradiated ones. Gamma radiation at an absorbed dose of 100 kGy extremely increases β-carotene

Table 2
Antioxidant activity of cork wastewater samples, non-irradiated and irradiated at different doses. In each row, different letters mean significant differences between average values ($p < 0.05$).

Samples	Antioxidant activity (EC_{50}, μg/mL)			
	0 kGy	20 kGy	50 kGy	100 kGy
DPPH scavenging activity	180 ± 12^a	151 ± 4^b	133 ± 2^c	119 ± 6^d
Reducing power	102 ± 0^a	90 ± 2^b	80 ± 1^c	68 ± 1^d
β - Carotene bleaching inhibition	207 ± 6^a	255 ± 4^b	150 ± 6^c	79 ± 4^d

Table 1
Cork boiling wastewater physico-chemical characterization before and after irradiation at different doses of gamma radiation. In each row, different letters mean significant differences between average values ($p < 0.05$).

Parameter	Average value ± Standard error			
	0 kGy	20 kGy	50 kGy	100 kGy
pH	5.14	5.28	5.26	5.12
COD (mgO_2 L^{-1})	2903 ± 264^a	3430 ± 0^a	3166 ± 264^a	3166 ± 264^a
BOD_5 (mgO_2 L^{-1})	394 ± 29^a	228 ± 14^b	41 ± 1^c	52 ± 6^c
Biodegradability index, BOD_5/COD	0.15	0.07	0.01	0.02
TP (mg gallic acid L^{-1})	680 ± 32^a	704 ± 24^a	$755 \pm 32^{a,b}$	823 ± 6^b
TSS (mg L^{-1})	134 ± 17^a	132 ± 7^a	$105 \pm 4^{a,b}$	74 ± 2^b

bleaching (62%) and increases significantly both DPPH scavenging activity and reducing power by approximately 33%. These results are in agreement with those reported by Madureira et al. (2013) with the same type of wastewater, which found a correlation between the antioxidant activity and the phenolic content, as expected. Although in a different matrix, Cabo Verde et al. (2013) observed the increase of phenolic content and antioxidant activity of raspberries after irradiation at 1.5 kGy. Also Pereira et al. (2014) mentioned the dependence of the antioxidant activity on the increase of total phenolic content in irradiated samples of borotutu (*Cochlospermum angolensis* Welw.), a medicinal plant commonly used in Angola for the treatment of liver diseases and for prophylaxis of malaria. Pereira et al. (2015) reported that gamma radiation at 10 kGy on *Ginkgo biloba* L. promoted its antioxidant potential.

The cork wastewater samples herein studied were subjected to an oxidative medium upon irradiation, which results from the continuous formation of the highly reactive HO radical, while the other important primary radical, the solvated electron, is partially scavenged by the oxygen naturally dissolved in the samples into the much less reactive superoxide radical anion. Therefore, it is reasonable to consider that HO radical plays a major role concerning the stable oxidation products profile after irradiation. This radical reacts by addition to unsaturated bonds and by hydrogen abstraction. Considering the presence of tannins and other complex phenolic compounds, the addition of OH radical to aromatic moieties increases the number of hydroxyl groups in the molecule structure. On the other hand, H abstraction can lead to single bonds cleavages which rise the concentration of smaller phenolic structures. These reaction pathways can occur sequentially and therefore contribute to the increase of both antioxidant activity and phenolic content. On the other hand, the increase in lipid peroxidation inhibition could be associated with the amount of tocopherols which are powerful lipophilic antioxidants and could be radiolytic products of cork wastewater samples. Previous work developed by Melo et al. (2011) regarding the degradation of gallic acid (one of the main compounds of cork cooking water) by irradiation suggested that stable radiolytic products from gallic acid solutions are phenolic compounds with aliphatic chains. Nevertheless, this hypothesis should be confirmed through the identification of these compounds.

3.3. Toxicity

Samples of diluted solutions of cork wastewater were investigated. The presented results will not include the dilutions of cork wastewater since they do not introduce any new relevant information. In fact, for lower concentrations of cork wastewater (1% and 10% (v/v)), no toxic effect was observed for the tested cells. For this reason, only results for the "real" cork wastewater sample (100% wastewater, non-diluted sample) will be presented.

3.3.1. Growth inhibition test

P. fluorescens and *B. subtilis* have the ability to persist in a wide range of environments, including soils, surface of plants (Scales et al., 2014) and aquatic habitats (Earl et al., 2008). These species can persist for many years in the environment and can tolerate extreme conditions. Due to these characteristics, *P. fluorescens* and *B. subtilis* were used as studied microorganisms in the growth inhibition test, to access the toxicity of cork cooking water samples.

Fig. 1 shows the results for cork wastewater treated by different gamma radiation doses on the growth inhibition of *P. fluorescens* and *B. subtilis*.

The obtained results indicated that cork wastewater samples were non-toxic for both studied bacteria. Comparing to the controls, no significant variation was observed for non-irradiated samples. It is suggested that the cork samples had no influence on the cellular development and viability. Paz et al. (2006) evaluated the toxicity of Buenos Aires Hospital wastewater samples and also detected that the effluents were non-toxic for *P. fluorescens*.

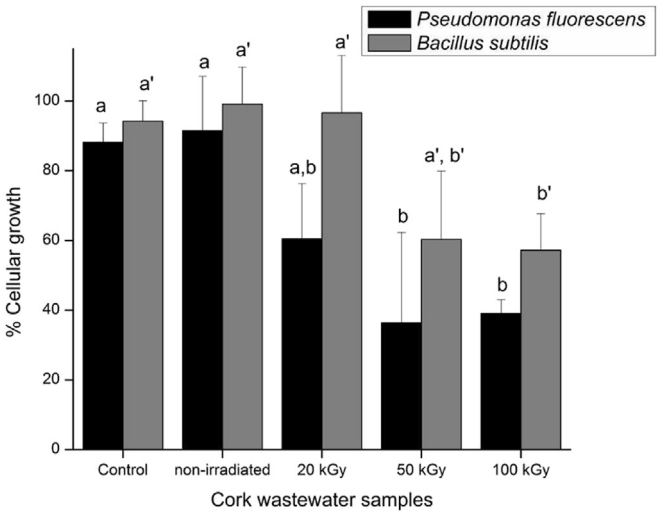

Fig. 1. Cellular Growth test of *Pseudomonas fluorescens* and *Bacillus subtilis* in the presence of cork wastewater non-irradiated and irradiated at several gamma radiation doses. Each bar graph represents the mean and 95% confidence interval of three separate experiments. For each microorganism, bars with different lowercase letter indicates a statistically significant difference from control at $p < 0.05$.

Considering the gamma radiation treatment, a significant decrease ($p < 0.05$) on the growth of *P. fluorescens* and *B. subtilis* was detected in the irradiated cork samples at 100 kGy, indicating a toxic effect of this sample. *P. fluorescens* appeared to be more sensitive to gamma radiation treated wastewater than *B. subtilis*. These results suggested that the radiolytic products formed at higher doses could be more toxic for *P. fluorescens* and/or *B. subtilis* than the parent ones.

As mentioned before, there are few studies about the ecotoxicity of cork boiling wastewater using different methodologies. Mendonça et al. (2007) studied the toxicity of these wastewaters to the bacterium *V. fischeri*, the crustacean *D. magna* and the plant *L. minor* and classified it as acutely toxic to the most sensitive species (*V. fischeri*). Gomes et al. (2013) also revealed a high acute toxicity of other cork industry wastewater to *V. fischeri*.

3.3.2. Cytotoxicity

Two different eukaryotic mammalian cell lines, human and mouse, were used to compare the potential cytotoxicity effects of cork compounds in different living beings (Ma et al., 2014). This study could be a preliminary approach to evaluate the applicability of cork wastewater extractable compounds in industries like food or cosmetics.

Fig. 2 presents the results of irradiated and non-treated cork wastewater cytotoxicity for both cell lines.

The evaluation of the cork wastewater cytotoxicity by cell viability measurement suggested a toxic behavior of cork wastewater samples on RAW264.7 and A549 cells. The presence of the cork water samples caused a significant ($p < 0.05$) reduction of the WST-1 mitochondrial metabolisation that reflects a lower number of viable cells/lower cellular activity relatively to the cells that were growing only in the presence of DMEM growth medium.

Regarding the wastewater irradiated samples, it was notorious an increase of the toxic effect on the studied eukaryotic cells

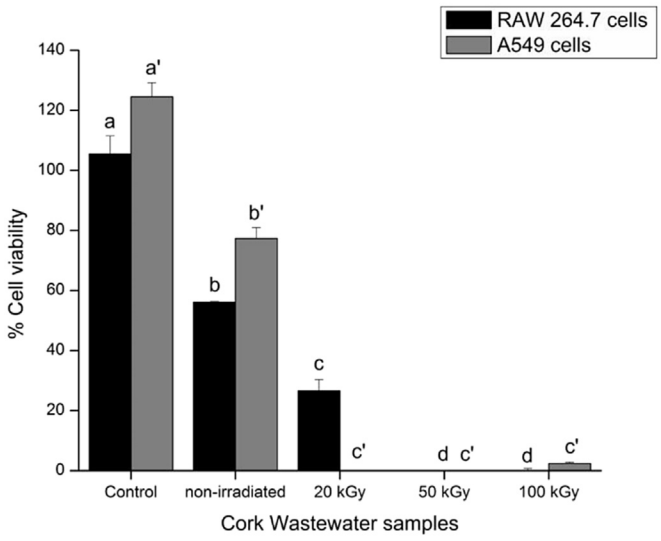

Fig. 2. Cellular viability of A549 and RAW264.7 cell lines in the presence of cork wastewater non-irradiated and irradiated at several gamma radiation doses. Each bar graph represents the mean and 95% confidence interval of three separate experiments. For each cell line, bars with different lowercase letter indicates a statistically significant difference from control at $p < 0.05$.

(significant decrease of cell viability, $p < 0.05$), especially for A549 cells. However, no significant ($p > 0.05$) differences were observed between the A549 cell viability percentages between the irradiated samples. For RAW 264.7 cells the toxic effect of irradiated cork wastewater samples was significantly different ($p < 0.05$) between 20 kGy and 50 and 100 kGy. This irradiation effect seemed to be less drastic when several dilutions of the samples were tested (data not shown), which could reveal that the concentration of cork wastewater compounds (irradiated and non-irradiated) is a critical factor for the toxicity effect on eukaryotic cells. A dilution 1:10 of cork wastewater in pure water was sufficient to reduce the toxicity of the original samples to a level similar to that obtained for the control of the cellular growth.

As far as we know, it is the first report on the use of these cell lines to assess the toxicity of cork wastewater with or without treatment. A recent study had published the use of RAW 264.7 cells to evaluate the cytotoxicity of the wastewater from a sewage treatment plant (Makene and Pool, 2015). The observed results were similar to the ones obtained in this work, showing that the water samples did not induce cytotoxic effects to the macrophage cells. Moreover, A549 human cells were used before to study the cytotoxicity of effluents from lab-scale sequencing batch reactors (SBRs) receiving engineered nanomaterials and no significant decrease of cell viability was observed when cells exposed to SBR effluents (Ma et al., 2014).

Taken together, the obtained results indicate that cork wastewater caused a significant reduction on the survival rate of the studied cells. When the samples are treated with gamma radiation, the cytotoxicity effect increased in a significant way, causing the death of all exposed cells. This behavior could be a consequence of the presence of cork radiolytic products and it is likely that these degradation products could be much more toxic than the initial cork wastewater components.

4. Conclusions

In this work, it was demonstrated that the compounds present in cork wastewater are non-toxic for prokaryotic cells (*Pseudomonas fluorescens* and *Bacillus subtilis*) but have a toxic behavior for eukaryotic cells (RAW264.7 and A549 cells). In fact, it is important to note that the toxicity of wastewaters depends on at least two factors: the studied organisms and the cork industry since the wastewaters components vary according to the different harvest locations. These results showed the importance of a combined evaluation of toxicity and chemical analysis to assess the environmental risk of industrial effluents.

The use of gamma radiation at doses of 100 kGy increased the cork wastewater toxicity for all eukaryotic and prokaryotic tested cells. This could suggest that the radiolytic products of cork wastewater seem to be more toxic than the parental ones and their on-going identification could bring a wide range of information to the toxicity studies (acute and chronic) of this type of complex samples.

Moreover, the results revealed that the antioxidant potential of effluent compounds increased with gamma radiation which could be relevant thinking in the application perspective of the cork wastewater extractable compounds in other industries like food or cosmetic ones.

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