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Original Article

Toxicological Response of *Saccharomyces cerevisiae* to Acetylsalicylic Acid Aqueous Solution Treated by Electron Beam Irradiation

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Abstract

Pharmaceuticals have drawn attention due to the potential of causing negative impacts on the population and ecosystems at ecological relevant concentrations. Among these contaminants, acetylsalicylic acid is a drug widely used in human medicine as an analgesic, antipyretic and in actively preventing blood platelet aggregation, which has been introduced into the environment continuously. Several technologies have been proposed for the removal of contaminants. Electron beam irradiation (EBI) has been applied as an alternative and clean technology for pollutant removal. Nevertheless, after any type of treatment there may occur the formation of more toxic byproducts, which may be detected by biological assays. The *Saccharomyces cerevisiae* yeast consists in simple eukaryotic model, widely used for the assessment of toxic effects on human cells and tissues. This work aims the acute toxicity assessment of treated acetylsalicylic aqueous solutions by EBI employing *Saccharomyces cerevisiae*. Toxicity assays were performed with *S. cerevisiae* and the results were compared to other aquatic organisms (*Vibrio fischeri* bioluminescent bacteria and *Daphnia similis* microcrustacean). The results showed low sensibility to the yeast when exposed to the anti-inflammatory, demonstrating an $EC50_{30min}$ of 815 mg L^{-1}, when compared with the bioluminescent bacteria ($EC50_{15min}$ = 38.48 mg L^{-1}) and the microcrustacean ($EC50_{48h}$ = 86.05 mg L^{-1}). Due to low acute toxicity, additional chronic assays were also performed with *D. similis*, demonstrating a $NOEC_{14days}$ (No Observed Effect Concentration) of 2.5 mg L^{-1}. Based on these results, toxicity data from chronic assays was utilized for PNEC estimation, and the highest concentration detected in Brazilian surface water was used to evaluate the worst-case scenario. The calculated risk quotient indicated a possible risk of acetylsalicylic acid to aquatic biota. After EBI treatment, increase in toxicity has been noted for all the evaluated organisms, indicating sensibility of the evaluated organism. This work demonstrated the feasibility of employing toxicity assays with the *Saccharomyces cerevisiae* yeast.

Keywords: Anti-inflammatory, Ecotoxicity, Electron Beam Irradiation, Pharmaceuticals, Risk assessment.

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INTRODUCTION

Urban and industrial growth has triggered the release of toxic compounds into the environment, causing negative impacts on the population and ecosystems (Nguyen *et al.,* 2017). Water pollution by pharmaceuticals has been acknowledged as an environmental problem. These compounds are designed to interact with specific molecular targets, which may affect non-target organisms in the ecosystems (Godoy and Kummrow, 2017). Also, these contaminants have been frequently detected in the environment at relatively low concentrations (μg to ng L^{-1}) with potential for inducing negative impacts on ecosystems and human health (Montagner *et al.*, 2019). In addition, they are incompletely metabolized and excreted unchanged. Furthermore, conventional water and wastewater treatment processes are unable for a reliable remove of some recalcitrant pharmaceuticals (Godoy and Kummrow, 2017).

Aspirin is commonly used in human medicine as an analgesic, antipyretic and in actively preventing platelet aggregation (Nunes *et al.,* 2015). Exposure to acetylsalicylic acid and its metabolic products may induce adverse effects to several species such as genotoxic effects, physiological and behavioral alterations in aquatic organisms (Gómez-Oliván *et al.,* 2014; Szabelak and Bownik, 2021).

Several technologies have been evaluated for the removal of pollutants from waters. Electron beam irradiation (EBI) is considered a clean process and an environmentally friendly alternative for degrading and removing toxicity pharmaceuticals from aquatic matrices (Boiani *et al.,* 2019; Silva *et al.,* 2016; Tominaga *et al.,* 2018, 2021)

Toxicity measurements are useful tools to evaluate the toxicity of generated byproducts (Rizzo, 2011), since biological assays allow not only to detect a wide spectrum of chemical contaminants, but also to predict effects produced by the interaction between mixtures of substances (Bosch-Orea *et al.,* 2017). There is a need for the development of simple, selective, and sensitive methods for monitoring the toxic chemicals in the environment, leading to new technologies, enabling economic and quick detection of toxic compounds along with the possibility of on-site monitoring. *Saccharomyces* yeasts are considered a very interesting organism for studies of toxicity. Several works have been proposing these organisms as a model for toxicity assessment (Dolezalova and Rumlova, 2014; Estève *et al.,* 2009; Hosiner *et al*., 2014; Hrenovic *et al,*. 2005; Keenan *et al.*, 2007; Knight *et al.*, 2004; Rumlova and Dolezalova, 2012).

This work aims the application of the yeast *Saccharomyces cerevisiae* as an organism model for acute toxicity assessment of treated acetylsalicylic aqueous solutions by EBI.

MATERIAL AND METHODS

Reagents

Acetylsalicylic acid was purchased from Labsynth (>99.5 %). All aqueous solutions were prepared by diluting the pharmaceutical in ultra-pure water (Millipore MiliQ). Acute experiments towards *Daphnia similis* and *Saccharomyces cerevisiae* were performed in three replicates. Toxicity assays with *Vibrio fischeri* were performed in duplicates.

Toxicity and risk assessment

Toxicity assays with yeast *Saccharomyces cerevisiae* were performed according to the adapted methodology of Dolezalova and Rumlova (2014), which is based on monitoring changes in the specific conductivity of yeast suspensions due to inhibition of fermentation under toxic conditions, after 30 minutes of exposure. Briefly, 20 mL containing the samples were transferred into test tubes containing 2.0 g of sugar. The control was carried out with sucrose solutions without the presence of chemical substances. After dilution, the specific conductivity of samples containing sucrose was measured. Then, 1.0 g of yeast was added to each tube and after 30 min the specific conductivity was measured.

Toxicity assays with *Vibrio fischeri* bacteria, following the recommendations of ABNT NBR 15411/2019, standard method. Initially, the lyophilized bacteria were reactivated with 1000 μL of reactivation buffer solution. According to the standard, samples with salinity < 20 need to be adjusted. The salinity of the samples was corrected using osmotic adjustment. Then, the control and the appropriate dilution series of the samples were prepared, using the diluent solution (sodium chloride 20 mg L^{-1} solution). The concentrations used were equivalent to 10.23 %; 20.47 %; 40.95 % and 81.90 %. Two sequences of cuvettes were placed in the Microbics® analyzer: the first with the samples containing the appropriate osmotic adjustment and the second row of cuvettes with 100 μL of reactivated bacteria. From the second sequence, luminescence values were obtained in order to calibrate the analyzer. Subsequently, measurements of I_0 (initial luminescence, without the sample) were obtained. Then, the respective samples were transferred to the reading cuvettes and after 15 minutes of exposure, the I_{15} values were recorded. The stock solutions of ASA (100 mg L^{-1}) were used in the assays.

Chronic toxicity assays with *Daphnia similis* were carried out according to OECD guideline 211 (OECD 2012) with modification of exposure time to 14 days, as proposed by (Vacchi *et al.,* 2016). One neonate (<24 h old) was transferred to a recipient containing 25 mL of the sample solutions. Ten control and ten treatment replicates were used for each concentration. The organisms were exposed

to five concentrations (1.25, 2.5, 5.0, 10 and 20 mg L^{-1}). The organisms were fed daily with *Raphidocelis subcapitata* and the test medium was renewed every two/three days. The organisms were maintained under controlled temperature (20 ± 1 °C) and photoperiod (16 h light). The number of living offspring produced by each parent animal was recorded.

The evaluation of the impact of the anti-inflammatory was calculated based on Technical Guidance Document on Risk Assessment (European Commission, 2003). The Risk Quotient (RQ) and based on the relationship between the effects obtained with the measured environmental concentrations (MEC), acquired from the literature and the estimated values of PNEC (Predicted No Effect Concentration). The chronic data was selected for the estimation of the PNEC and the RQ. The highest measured environmental concentration (MEC) value of the anti-inflammatory in Brazilian surface waters was used for the risk assessment to verify the real worst-case scenario. The PNEC was estimated by the acute toxicity data divided by an assessment factor (AF = 100) of 100 (NOEC obtained from chronic assays with Daphnia).

Statistical analysis

Statistical analysis for yeast assays consisted of the F-test and the t-test. The data were analyzed by regression analysis. The $EC50_{(30min)}$ values were defined as the concentration of toxicants, which cause a 50 % change of the average corrected specific conductivity in comparison with the average corrected specific conductivity of the control sample. Both the F-test and the t-test were employed, with a confidence level of 0.05. It was found that a decrease of about 10 % of the corrected mean specific conductivity is statistically significant and represents the lowest reliable concentration detected of the toxic agent by this assay (Dolezalova and Rumlova, 2014).

The results of bioluminescent *V. fischeri* were expressed in $EC50_{15min}$ (Median Effective Concentration) obtained by linear regression. For the irradiated samples, the results were expressed in toxicity factor (TF), which is not calculable and was expressed by the value of the dilution factor corresponding to the highest concentration of the sample in which is observed no inhibition greater than 20 %.

For chronic assays performed with *D. similis*. the EC50 values were determined using regression analysis, by applying a three-parameter-logistic-fit (sigmoidal logistic model). A one-way analysis of variance (ANOVA) was used to verify differences between treatments and controls of the chronic assays. When significant ($p < 0.05$), ANOVA was followed by a Dunnett's post hoc test. The NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) were calculated for chronic assays with this method.

Irradiation Procedure

The liquid samples containing aqueous solutions of pharmaceuticals were irradiated in batches in a Dynamitron® Industrial Electron Accelerator with a power of 37.5 kW. The energy was fixed at 1.4 MeV, varying only the electric current. Absorbed doses of 2.5 kGy was applied to enable the operating cost and minimize process energy. During irradiation, the samples were kept at room temperature, in a rectangular container (Pyrex®), with a volume of 246 mL, to ensure 4 mm of thickness, and thus, guarantee the maximum penetrability, as described in previous works (Silva *et al.,* 2016; Tominaga *et al.,* 2018, 2021).

RESULTS AND DISCUSSION

Toxicity assessment of acetylsalicylic acid

Acute toxicity data for the three species are shown in Table 1. *V. fischeri* was the most sensitive, while *Daphnia similis* presented intermediate sensitivity to the anti-inflammatory (Table 1). Although the $EC50_{15min}$ of *V fischeri* corroborates with Calleja *et al.,* (1994), which have obtained a value of 26.1 mg L^{-1} for the bacterium *V. fischeri*. Di Nica *et al.*, (2017) reported lower aspirin toxicity ($EC50_{15min}$ of 267.6 mg L^{-1}).

The yeast showed the least sensitivity to ASA (Table 1). Nevertheless, the results indicated that there were significant alterations in the fermentation between the control and the samples, denoting the presence of toxic effects in all tested concentrations (Figure 1). Previous studies have shown that aspirin can cause apoptosis in some wild-type strains of the yeast *Saccharomyces cerevisiae* (EG103, EG110 and EG 118) depending on the available carbon source, occurring mainly in glucose-containing medium (Balzan *et al.,* 2004). It occurs since acetylsalicylic acid impairs the synthesis and transport of acetyl coenzyme A into the mitochondria, and thus affects energy production (Farrugia *et al.,* 2019).

Overall, the obtained results corroborate studies with different trophic levels. Kusk *et al.*, (2018) reported an $EC50_{48h}$ of 241 mg L^{-1} for the algae *Raphidocelis subcapitata* and Cleuvers (2004) estimated an EC50 of 106.7 mg L^{-1} for the alga *Scenedesmus subspicatus*.

According to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) (2010) acetylsalicylic acid is classified as harmful to aquatic life (Acute Toxicity, Category 3, $10 < EC50 < 100$ mg L^{-1}, for crustaceans). Thus, additional chronic assays were necessary for assessment of sub-lethal effects. Chronic assays with *D. similis* ranged up to 1 order of magnitude from the acute assay (Table 1). The obtained values for ASA corroborate previous

works described in the literature. $CL50_{48h}$ of 88.31 mg L^{-1} and 88.33 mg L^{-1} were reported for *D. magna* after exposure to ASA (Cleuvers, 2004; Gómez-Oliván *et al.,* 2014). Marques *et al*., (2004) reported $LOEC_{21days}$ and $NOEC_{21days}$ of 1.8 and 1.0 mg L^{-1}, respectively, for *D. magna* and *D. longispina*, demonstrating a significant reduction in reproduction.

The ecotoxicity data used for deriving the standards are shown in Table 3. The calculated RQs indicated aspirin has a possible risk (0.1 < RQ < 1.0). Acetylsalicylic acid has been detected at higher concentrations at surface water in Brazil (0.012 to 20.96 µg L^{-1}), implying possible risk or high risk to the aquatic biota (Montagner *et al.,* 2019; Sodré *et al.*, 2018).

Table 1 – Acute toxicity data (Effect concentration at 50 % (EC50%) obtained in the tests carried out towards different organisms (*Vibrio fischeri* bioluminescent bacteria, *Saccharomyces cerevisiae* yeast and *Daphnia similis* water flea) for evaluating the toxicity of acetyl salicylic acid.

Test organism	Toxicological endpoint	Ecotoxicity data (mg L^{-1})
Vibrio fischeri	$EC50_{15min}$	38.5 ± 4.5
Saccharomyces cerevisiae	$EC50_{30min}$	815 ± 119
Daphnia similis	$EC50_{48h}$	86.1 ± 4.6[a]

[a] Tominaga et al. (2021)

Table 2 – Chronic toxicity data (Effect concentration at 50 % (EC50), Lowest Observed Effect Concentration (LOEC), and No Observed Effect Concentration (NOEC)) obtained the chronic tests carried out for evaluating the toxicity of acetyl salicylic acid toward *D. similis*.

Test organism	Toxicological endpoint	Ecotoxicity data (mg L^{-1})
Daphnia similis	$EC50_{14days}$	13.1
	$LOEC_{14days}$	5.00
	$NOEC_{14days}$	2.50

Table 3 - Risk quotient (RQ) based on maximum measured environmental concentrations (MEC) reported in the literature for the detection and quantification of acetylsalicylic acid in Brazilian fresh surface water. The predicted non-effect concentration (PNEC) value was estimated by the application of an assessment factor (AF) to the lowest NOEC value obtained from chronic assays.

MEC (µg L^{-1})	Reference	NOEC (µg L^{-1})	AF	PNEC	Risk Quotient
20.96	Montagner & Jardim (2011)	2500	100	25	0.84

It is important considering that pharmaceuticals do not occur isolated in aquatic environments. The risk of pharmaceutical mixtures in the environment can overcome the risk of each individual compound, and the individual environmental quality standards do not necessarily guarantee protection against adverse mixture effects (Backhaus, 2016; Godoy *et al.,* 2018)which calls specific attention to the fact that monitoring surveys routinely find complex pharmaceutical mixtures in various environmental compartments. However, although the body of evidence on the ecotoxicology of pharmaceutical mixtures is quite consistent, the current guidelines for the environmental risk assessment of pharmaceuticals often do not explicitly address mixture effects. Data availability and acceptable methods often limit such assessments. A tiered approach that begins with summing up individual risk quotients, i.e., the ratio between the predicted or measured environmental concentration and the predicted no effect concentration (PNEC. Previous works have shown both antagonistic and synergistic features depending on the applied amounts and concentration of pharmaceuticals on mixture acute assays (Tominaga *et al.,* 2022). The authors also identified hormetic effects at low concentrations of a quaternary mixture assessed by chronic assays with daphnids, indicating that the effects of individual pharmaceuticals can underestimate the risk level of these contaminants in the environment.

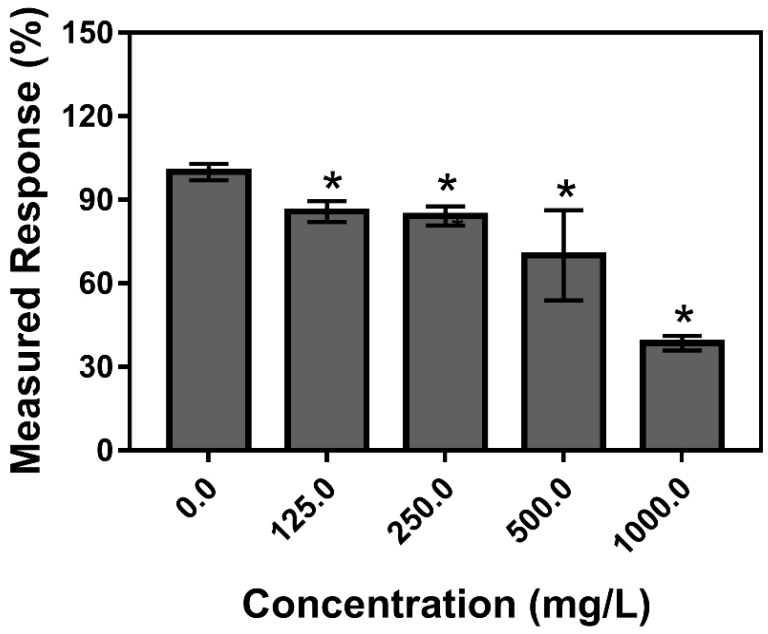

Figure 1 –Dose-response curve plotting percent inhibition of fermentation and deviation standard of *Saccharomyces cerevisiae* yeast exposed to acetylsalicylic acid with 30 min exposure. The asterisks (*) indicate significant different compared to the control.

Electron Beam Irradiation

Increase in toxicity has been demonstrated after the treatment for all the evaluated organisms, indicating the possible application of yeast as an organism model for rapid toxicity assessment (Table 4). After the degradation process, the formation of more toxic byproducts than the parental compound may occur. Therefore, toxicity measurements are useful tools to evaluate the toxicity of degradation products. Previous works have shown increase of toxicity of anti-inflammatories treated by ionizing radiation, such as acetylsalicylic, paracetamol and ibuprofen (Illés *et al.,* 2013; Szabó *et al.,* 2012, 2014). Hydroxyl radicals were shown to degrade these molecules readily, and first degradation products were hydroxylated derivatives in all cases. Due to the by-products, among them hydrogen peroxide, the toxicity first increased and then decreased with the absorbed dose. With prolonged irradiation complete mineralization was achieved. Using radiolytic experiments hydroxyl radical (main reactant in advanced oxidation processes. According to Daescu *et al.,* (2021), the intermediate compounds of acetylsalicylic formed by the photocatalytic oxidation process or different mechanisms such as hydrolysis, electrolytic addition, electron transfer, decarboxylation reaction, aromatic ring opening and radical reaction, are more toxic than the parent compound.

EBI is an oxidative advanced process based on the water radiolysis, which produced both oxidative species (hydroxyl radicals, •OH) and reductive species hydrogen atoms H• and aqueous electron (e^-_{aq}), that are able to interact and promote the oxidation, reduction, dissociation or degradation of various organic pollutants (Buxton, 2008; Capodaglio, 2017). Tominaga *et al.,* (2021) has demonstrated that low doses (1 to 5 kGy) are able to degrade acetylsalicylic acid to concentrations below limit detection (70.4 μg L^{-1}), producing degradation products.

Table 4 – Acute toxicity assessed by *V. fischeri*, *D. similis* and *Saccharomyces cerevisiae* for untreated and ASA irradiated at 2.5 kGy. Initial conditions: $[ASA]_0$ = (9.48 ± 1.12) mg L^{-1}; pH = 6.24 ± 0.31.

Test organism	Doses (kGy)	
	0.0	2.5
Vibrio fischeri	1 TF	2 TF
Daphnia similis	2 TF [a]	4 TF [a]
Saccharomyces cerevisiae	Non-toxic	Toxic

[a] Tominaga et al. (2021). TF: Toxic Factor

In addition, under aerated conditions, it may occur hydrogen peroxide formation, which is detrimental for biological assays (Illés *et al.,* 2017; Szabó *et al.,* 2014; Tominaga *et al.,* 2021). Szabó *et al.,* (2014) and Tominaga *et al.,* (2021) reported significant toxicity reduction in treated solutions after H_2O_2 removal using catalase, also reporting low remaining toxicity for *V. fischeri* and *D. similis*. Therefore, the combination of technologies can increase contaminant removal efficiency to safer disposal levels. For instance, Changotra *et al.*, (2020) investigated the application of a hybrid sequential treatment process of coagulation, EBI and biological treatment of real pharmaceutical industrial wastewater. The results indicated that the sequential treatment route lead to synergistic degradation and detoxification of the pharmaceutical wastewater streams with overall improved chemical oxygen demand and total organic carbon removal efficiencies. Besides, cytotoxic evaluation revealed that irradiated wastewaters did not exhibited toxicity against the selected microorganisms (*E. coli*, *B. subtilis* and *P. aeruginosa*).

CONCLUSION

This study presented the feasibility for the application of *S. cerevisiae* as a possible test-organism for rapid biological assays. Different ecotoxicity data were compared, indicating that the bacterium was more sensitive to the pharmaceutical, while the yeast was the least sensitivity. The results reinforce the importance of using different trophic level organisms, in order to guarantee suitable protection to the different non-target organisms in nature and keep a necessary quality of the aquatic environment. Increased toxicity effects were noted after EBI for all the species, indicating the possibility of the application of *S. cerevisiae.*

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data, associated metadata, and calculation tools are available from corresponding author Flavio K Tominaga (fktominaga@gmail.com)

CREDIT AUTHOR STATEMENT

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