Orbital
The electronic journal of chemistry

the electronic journal of chemistry

Full Paper | http://dx.doi.org/10.17807/orbital.v15i3.17993

Combined Toxicity of Methylparaben and Propylparaben in *Artemia salina* and *Allium cepa* Applying Experimental Design

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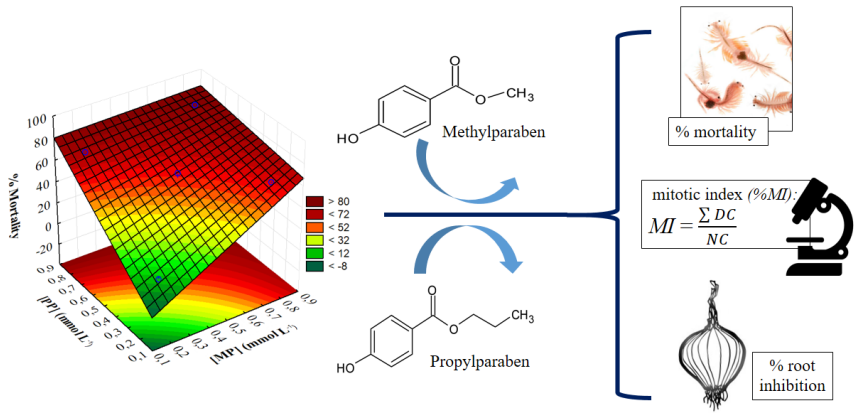
Parabens are used as preservatives in sanitizers and cosmetic products causing environmental concern, because presented potential as endocrine disrupters. Among these compounds, the most used are methylparaben and propylparaben. Thus, a study was proposed to evaluate the interaction between different concentrations (mmol L^{-1}) of the variables methylparaben [MP] and propylparaben [PP], against the acute toxicity of the microcrustacean *Artemia salina* (*A. salina*) and *Allium cepa* (*A. cepa*) applying the 2^2 factorial design with an added center point. Responses were used: percent *A. salina* mortality (% mortality), *A. cepa* root growth inhibition (% root inhibition) and mitotic index (%MI). For *A. salina*, after 72 hours of exposure with the combination of concentration ([MP] and [PP] = 0.8 mmol L^{-1}) caused an 80% mortality. While, *A. cepa* a high cytotoxicity was observed with the mixture of Parabens, exhibiting 72.3% root growth inhibition at [MP] = 1.2 mmol L^{-1} with [PP] = 1.2 mmol L^{-1}. In contrast, for response %MI at [MP] = 0.3 mmol L^{-1} with [PP] = 0.3 mmol L^{-1}, 2.5 %MI with 36% inhibition. In this context, parabens demonstrated high toxicity for *A. salina* and cytotoxicity for *A. cepa*, based on the interaction with the effect of the concentrations.

Graphical abstract



Keywords

Parabens
Artemia salina
Allium cepa
Acute toxicity
Experimental design

Article history

Received 02 Feb 2023
Revised 03 Jul 2023
Accepted 11 Sep 2023
Available online 11 Oct 2023

Handling Editor: Adilson Beatriz

1. Introduction

Preservatives in pharmaceuticals and personal care products (PPCPs) are a class of substances that cause environmental concern due to their harmful effects on the ecosystem, because they are considered persistent

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compounds. These compounds are more resistant to being degraded in the environment and /or the inefficient destruction of municipal water treatment plants that increase their concentration in nature [1,2, 3, 4, 5].

Among PPCPs, Alkyl esters of p-hydroxybenzoic acid (parabens), since are widely used in cosmetics, sanitizers, and pharmaceutical products in general. The most commonly used parabens as preservatives are methylparaben and propylparaben. Studies indicate that these substances have estrogenic activities for aquatic organisms and mammals, therefore considered endocrine disruptors [1, 3, 6, 7, 8].

The commercial form of Nipagin is methyl 4-hydroxybenzoate, also known as methylparaben (MP). This compound with molecular formula $C_8H_8O_3$, a molar mass equal to 152.15 g mol^{-1}, and molecular structure shown in Fig 1a), has been detected in drinking water samples at a concentration of 12 ng L^{-1}, in shallow statuary in Portugal in the concentration range of 2.1–51 ng L^{-1}[6]. Other authors detected methylparaben in surface water at a concentration of 0.262 µg L^{-1} in the State of Rio Grande do Sul, South Brazil [9]. While the commercial product Nipazol is another widely used Paraben, also known as propylparaben (propyl 4-hydroxybenzoate). This preservative with molecular formula $C_{10}H_{12}O_3$, molar mass equal to 180.18 g mol^{-1} and a molecular structure shown in Fig 1b). Propylparaben (PP) has also been detected and quantified in drinking water at a concentration of 9 ng L^{-1} and in the concentration range of 7–9 ng L^{-1} in shallow statuary [6].

Another study carried out in surface waters in São Paulo, Brazil, more specifically in the river in Mogi Guaçu, quantified higher concentrations of 27.5 and 2.8 µg L^{-1} of methylparaben and propylparaben, respectively [10].

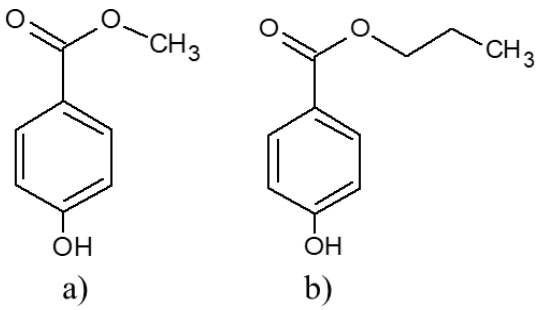

Fig. 1. Molecular structure a) methylparaben and b) propylparaben.

Methylparaben and propylparaben have a common characteristic, they cause an increase in vitellogenin protein (VTG) in male fish and, therefore are considered endocrine disruptors [8,9]. Puerta et al (2020) [9] reported that methylparaben produced inhibition of green algae growth. Other authors like Di Poi et al (2018) [11] correlated the MP with increased toxicity of the Glyphosate herbicide about Green Algae. In addition to these researchers, the assessment of synergistic effects of toxicity between Parabens was also questioned by Derisso et al (2020) [12] and Feng et al (2019) [13]. Nana et al. (2022) [14] carried out toxicity studies with a mixture of methyl-, ethyl-, and propylparabens and observed synergistic and antagonistic effects against luminescent bacteria *Vibrio qinghaiensis sp.* Q67.

In this context, studies with the objective of combined substances against toxicity are relevant, being the starting point of the work. This study of the interaction of methylparaben and propylparaben preservatives was carried out with a 2^2 factorial design with added center point employed to bioassays for the microcrustacean *Artemia salina* (*A. salina*) and *Allium cepa* (*A. cepa*).

2. Material and Methods

2.1 Reagents

Bioassays were performed with Methylparaben (Nipagin) and Propylparaben (Nipazol) reagents (98% Chimia Limited – China). The synthetic seawater used for the test with *A. salina* was commercial. Meanwhile, reagents for the preparation of solutions were purchased from Synth: hydrochloric acid (37% P.A.), glacial acetic acid (100% P.A.), and ethyl alcohol (99.5% P.A.). For the study with *A. cepa* the dye orcein P.A. was used from the Dinâmica.

2.2. Acute toxicity to *Artemia salina*

Toxicity tests were performed with larvae of the microcrustacean *A. salina* ecloded in synthetic seawater (32 g L^{-1}), pH 8-9, aerated for 48 h. Tests were performed in triplicate (10 individuals per replicate), at 20 ± 2 °C, with a 16 h light and 8 h dark photoperiod for 72 hours in a static system with 10 mL solution for each test. Dead larvae were counted for each test. The experiments were carried out for five dilutions with synthetic seawater (100, 50, 25, 12.5, and 6.25 %, v/v), where the mortality value for application in the experimental design was in the solution at 100%, i.e., without dilution [15, 16, 17].

2.3. Ecotoxicity evaluation to *Allium cepa*

The test with *A. cepa* employed onion bulbs obtained commercially and were acclimated in tap water for 24 hours. Then exposed to the solutions for 48 hours with six replicates for each Parabens mixture. The response evaluated was two parameters: root length (inhibition of root growth) and mitotic index (alterations in cell division). Additionally, it was maintained a negative control group with distilled water and measured the length of the three longest roots (To calculate their average length) of each onion. Thus, it was possible to compare the test with the negative control [18, 19, 20].

Cytotoxicity produced by Parabens can be determined by the mitotic index. For this analysis, roots were cut and suspended in Carnoy solution for 24 hours and thereafter preserved in ethanol 70%. Then Root tips were hydrolyzed in HCl (1 mol L^{-1}) under heating at 60° C for 10 min, followed by the addition of orcein (2%) and crushing against microscopy slides. The percentage mitotic index (%MI) for each bulb was calculated with a ratio of the number of dividing cells for 1,000 cells based on [18, 19, 20, 21].

2.4 Factorial design

Experimental design is a statistical strategy for reducing, organizing, and studying the interaction between variables with the aid of the response surface methodology (RSM) [16, 22]. Thus, a factorial design with an added center point based on the RSM was employed to evaluate the interaction between methylparaben and propylparaben against acute toxicity in *A. salina* and *A. cepa*. To evaluate this interaction used a response: percent *A. salina* mortality (% mortality), percent *A. cepa* root inhibition (% root inhibition), and percent mitotic index (%MI); with two independent variables: concentrations of methylparaben ([MP] (mmol L^{-1})) and propylparaben ([PP] (mmol L^{-1})). A 2^2 factorial design was then constructed, with a

total of seven trails four cube points and a triplicate at the center point [16, 22]. The concentration values used were based on Herrero et al (2012) [23]. Table 1 shows are variables and levels with the concentration range of 0.2 to 0.8 mmol L^{-1} for *A. salina* and from 0.1 to 1.2 mmol L^{-1} for *A. cepa*. For to generate experimental matrix was employed Statistica 10 software (StatSoft, Tulsa, USA). However, the bioassay *A. cepa*, were carried out in two experimental design with different concentrations.

Table 1. Levels of the 2^2 factorial design with an added center.

Bioassay	Factorial design	Variables	Levels		
			-1	0	+1
Artemia salina		**[MP] (mmol L^{-1})**	0.2	0.5	0.8
		[PP] (mmol L^{-1})	0.2	0.5	0.8
Allium cepa	**First**	**[MP] (mmol L^{-1})**	0.4	0.8	1.2
		[PP] (mmol L^{-1})	0.4	0.8	1.2
	Second	**[MP] (mmol L^{-1})**	0.1	0.2	0.3
		[PP] (mmol L^{-1})	0.1	0.2	0.3

3. Results and Discussion

3.1. Factorial design with an added center point

Table 2 summarizes the results obtained for the seven experiments with a factorial design with added center point chosen for the independent variables (Table 1). Based on the response surface methodology, the following polynomial Equation 1 was deduced to describe the interaction between independent and dependent variables:

$$Y = \beta_0 ++ \sum_{1 \le i \le j}^{k} \beta_{ij} X_i X_j \quad (1)$$

Where k represents the number of variables, *Y* is the dependent variable (percentage *A. saline* mortality, *A. cepa* root inhibition or mitotic index), and β_0, β_i, β_{ij}, denote the regression coefficients for the linear effects related to the linear X_i and X_iX_j interaction terms.

3.2. Percentage *A. salina* mortality and *A. cepa* root inhibition response

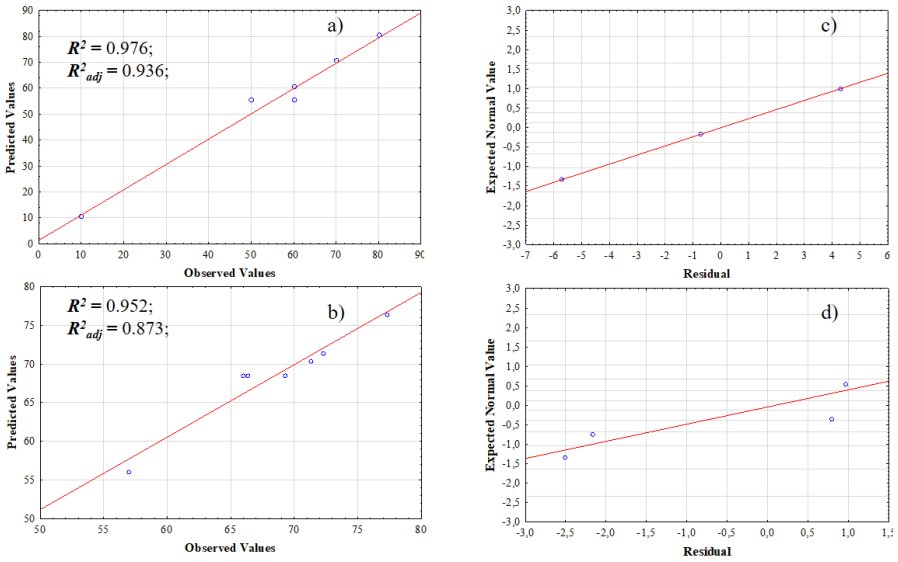

Fig. 2. Comparison of predicted and observed values for the percentage of a) % Mortality; and b) % root inhibition. Residual plots for the responses of the percentages of c) % Mortality; and d) % root inhibition.

Table 2. Observed and predicted values of the percentage of mortality, *A. cepa* root inhibition, and mitotic index, using different combinations factorial design with added center point.

Exp.*	[MP] (mmol L^{-1})	[PP] (mmol L^{-1})	% Mortality		[MP] (mmol L^{-1})	[PP] (mmol L^{-1})	% root inhibition		[MP] (mmol L^{-1})	[PP] (mmol L^{-1})	% root inhibition		% MI	
			Obs.	Prev.			Obs.	Prev.			Obs.	Prev.	Obs.	Prev.
1	0.20	0.20	10.0	10.7	0.40	0.40	57.0	56.0	0.10	0.10	22.0	23.1	8.60	8.77
2	0.80	0.20	60.0	60.7	1.20	0.40	77.3	76.3	0.30	0.10	30.0	31.1	6.70	6.87
3	0.20	0.80	70.0	70.7	0.40	1.20	71.3	70.3	0.10	0.30	14.0	15.1	7.50	7.67
4	0.80	0.80	80.0	80.7	1.20	1.20	72.3	71.3	0.30	0.30	36.0	37.1	2.50	2.67
5	0.50	0.50	60.0	55.7	0.80	0.80	69.3	68.5	0.20	0.20	29.7	26.6	6.60	6.5
6	0.50	0.50	60.0	55.7	0.80	0.80	66.34	68.5	0.20	0.20	25.6	26.6	7.20	6.5
7	0.50	0.50	50.0	55.7	0.80	0.80	66.0	68.5	0.20	0.20	29.4	26.6	6.40	6.5

*Experiments (Exp.).

Predicted responses by factorial design with added center point summarized in Table 2 were generated as arithmetic averages with ±95% confidence limits, and are shown in Fig. 2a and 2b. Correlation coefficients (R^2) and adjusted correlation coefficients (R^2_{adj}) were determined from the observed and predicted values, respectively. It was observed linear relationships presented good R^2-values between 0.976 and 0.936, as well as adjusted correlation coefficients (R^2_{adj}) between 0.952 and 0.873, for % mortality and % root inhibition, respectively. These values predicted by factorial design with an added center point were generated as arithmetic averages for confidence limits of ±95% (Table 2). Moreover, residuals correspond to the difference between predicted and observed results. Therefore, Fig. 2c and 2b evidence that the corresponding expected normal value varies linearly with the residuals, i.e., describing appropriately the responses [16, 22]

The influence of each independent variable, their interactions, and curvature can be studied by analysis of variance (ANOVA). Table 3 shows the ANOVA results of the linear regression model obtained for percentages of mortality and root inhibition. From the factorial design, table was obtained the sum of squares (SS), which measures the influence of the corresponding variable on the variation of the response values. Moreover, the degrees of freedom (df) corresponds to the number of columns of responses obtained, and the ratio between SS and df is the related mean of the squares (MS). For a probability level 95% a high F-values and low *p*-values (lower than 0.05) are evidence that the statistical significance for a model [24-26]. Thus, based on Table 3, it was not statistically significant curvature with lowed F-value equal to 0.143 and 2.66 with high p-value of 0.742 and 0.244 for percent mortality and root inhibition responses, respectively. This behavior signaling was not necessary to be applied a central composite design (CCD) model for the study [26, 27].

Table 3. ANOVA table results for factorial design 2^3 obtained for percent mortality and root inhibition responses.

Response	Factor	SS	df	MS	F-value	*p*-value
%mortality	Curvature	4.762	1	4.762	0.143	0.742
	[MP] (mmol L^{-1})	900.000	1	900.000	37.80	0.0086
	[PP] (mmol L^{-1})	1600.00	1	1600.00	67.20	0.0037
	[MP] *by* [PP]	400.00	1	400.00	16.80	0.0026
	Error	71.43	2	23.810		
	Total SS	2971.43	6			
% root inhibition	Curvature	8.77	1	8.77	2.66	0.244
	[MP] (mmol L^{-1})	113.42	1	113.42	22.15	0.018
	[PP] (mmol L^{-1})	21.62	1	21.62	4.22	0.132
	[MP] *by* [PP]	93.12	1	93.12	18.19	0.023
	Error	15.36	2	5.12		
	Total SS	243.52	6			

SS: Sum-of-Square; df: degree of freedom; MS: Mean Square.

3.3 Percent *A. salina* mortality response

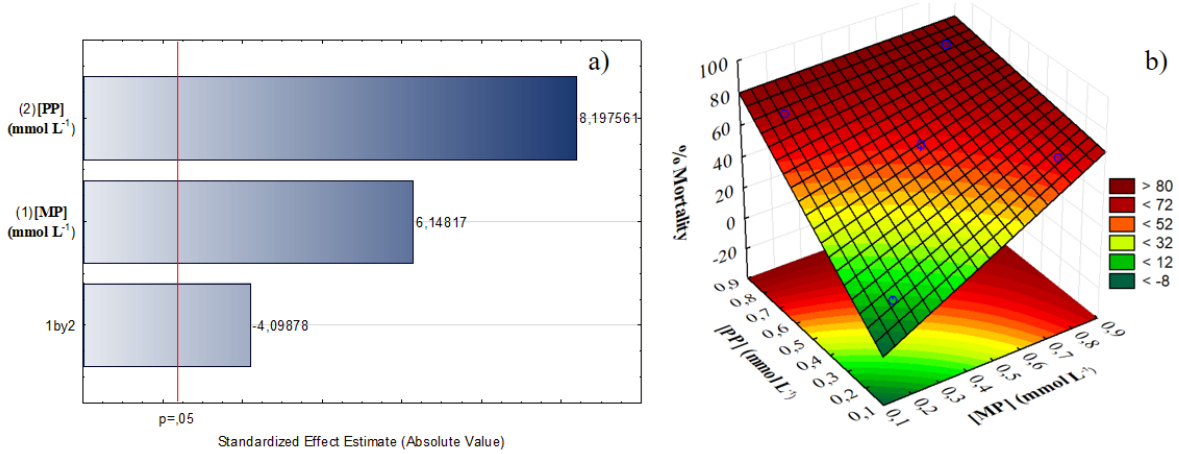

Fig. 3. a) Pareto chart; b) Response surface plot for percent *A. salina* mortality ([MP] *vs* [PP]).

The variables concentrations [MP] (mmol L^{-1}), [PP] (mmol L^{-1}), and the relationship between them were statistically significant ($p<0.05$), according to the Pareto chart (Fig. 3a). The surface plot based on the dependent variable (%mortality) was generated (Fig. 3b).

Fig. 3b reveals that the highest concentration of Parabens ([MP] = 0.8 mmol L^{-1} and [PP] = 0.8 mmol L^{-1}) caused 80% mortality. In contrast, the experimental ([MP] = 0.2 mmol L^{-1} and [PP] = 0.2 mmol L^{-1}) observed a decrease in neonate mortality, resulting in 10%. This behavior results that the concentration of both Parabens was decisive to obtain a high mortality rate, due to a high acute *A. salina* toxicity. The concentration combinations: (*i*) concentration of [MP] = 0.2 mmol L^{-1} with [PP] = 0.8 mmol L^{-1}, produced 70% of mortality; and (*ii*) concentration of [MP] = 0.8 mmol L^{-1} with [PP] = 0.2 mmol L^{-1}, generated 60%. In both experiments, it was obtained close values. Therefore, the interaction between methylparaben and propylparaben against acute toxicity was

based on the concentration and not on the chemical characteristic of the Parabens. Nana et al (2012) [14], observed an effect of the concentration of methylparaben and propylparaben preservatives against the acute toxicity of luminescent bacteria Vibrio qinghaiensis sp. Q67.

Other observations for Fig. 3b, were surface plot contour indicated that the percent mortality effects were similar under the high of the Parabens concentration and exhibiting high acute toxicity to microcrustaceans.

Equation 2 shows the estimated regression coefficients generated empirical model of percent mortality efficiency considering variables and mutual relationships.

$$Y_{\%mortality} = 55.7 + 30X_{[MP]} + 40X_{[PP]} - 20X_{[MP]}\,X_{[PP]} \quad (2)$$

Where $X_{[MP]}$ and $X_{[PP]}$ represent methylparaben and propylparaben concentration variables, respectively, and $Y_{\%mortality}$ is the percentage of mortality response.

The effect of variables is reflected in the values and signs of the estimated regression coefficients. Positive coefficients indicate that the toxicity efficiency improves with increasing concentrations of the respective variable. While negative coefficients indicate that toxicity efficiency is improved at lower concentrations. Furthermore, the synergistic effect in the interaction between variables shows a positive coefficient, unlike of antagonistic effect with a negative coefficient [28, 29, 30].

Based on Equation 2, *A. salina* mortality efficiency was found to improve with increasing methylparaben and propylparaben concentration as shown by the positive coefficients. This behavior results in a higher amount of preservatives introduced in the samples. It is important to highlight that the Parabens have acute toxicity for microcrustaceans, green algae, and fish [8, 9]. The relationship between [MP] and [PP] concentrations, was observed as a negative coefficient. Thus, an antagonistic effect was evidenced in the experiments: (*i*) concentration of [MP] = 0.8 mmol L^{-1} with [PP] = 0.8 mmol L^{-1}, produced 80% of mortality; while (*ii*) concentration of [MP] = 0.2 mmol L^{-1} with [PP] = 0.2 mmol L^{-1}, generated 10%. Decreasing both Parabens concentration simultaneously did not affect efficiency in the same in the toxicity, because the model was based on death *A. salina* efficiency.

3.4 First factorial design: Percent *A. cepa root inhibition* response

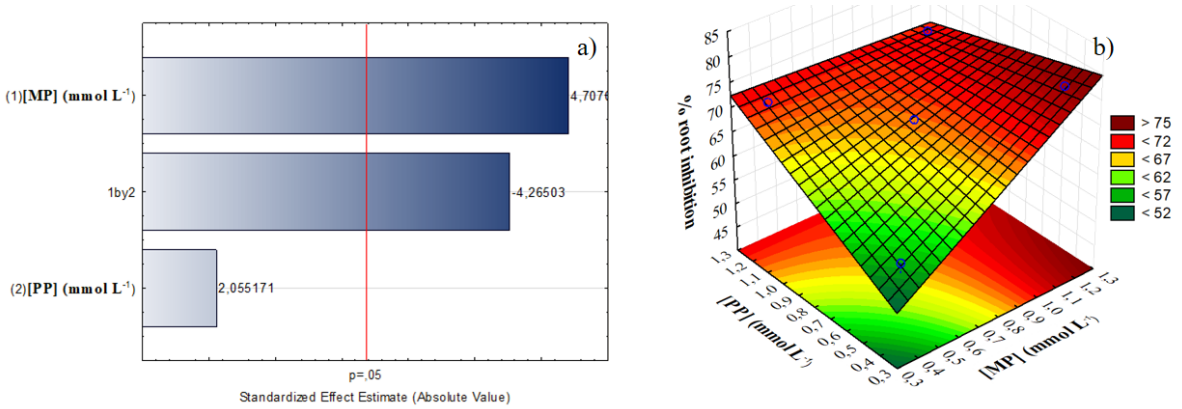

Fig. 4. a) Pareto chart; b) Response surface plot for percent *A. cepa root inhibition* ([MP] *vs* [PP]).

The Pareto chart of Fig. 4a obtained for the percent root inhibition highlights the statistical significance ($p<0.05$) of the methylparaben concentration and the relationship between [MP] (mmol L^{-1}) with [PP] (mmol L^{-1}). The surface plot based on the dependent variable (% root inhibition) was generated (Fig. 4b).

The results showed that the highest concentration ([MP] = 1.2 mmol L^{-1} and [PP] = 1.2 mmol L^{-1}) inhibited 72.3% of the root growth of the *A. cepa* (Fig. 4b). Decreasing the percent of inhibition of *A. cepa* in the lowest levels ([MP] = 0.4 mmol L^{-1} and [PP] = 0.4 mmol L^{-1}), that it exhibited 57%. The concentration of both Parabens contributed to obtaining high acute toxicity for *A. cepa* a highly inhibitory effect on root growth. The concentration experimental combinations: (*i*) concentration of [MP] = 1.2 mmol L^{-1} with [PP] = 0.4 mmol L^{-1}, obtained 77.3% growth inhibition; and (*ii*) concentration of [MP] = 0.4 mmol L^{-1} with [PP] = 1.2 mmol L^{-1}, inhibited 71.3%, showing the close values. However, for the model the methylparaben concentration increased the cytotoxicity, consequently the *A. cepa* root growth.

The generated empirical model for $Y_{\%inhibition}$ expressed as a function of the concentration variable and mutual relationship above defined was given by Equation 3:

$$Y_{\%inhibition} = 68.5 + 10.7X_{[MP]} - 9.7X_{[MP]}\,X_{PP]} \quad (3)$$

Where $X_{[MP]}$ and $X_{[PP]}$ represent methylparaben and propylparaben concentration variables, respectively. While $Y_{\%inhibition}$ is the percentage root inhibition response for the first factorial design.

The positive coefficient for the methylparaben concentration variable is indicative of an improvement of the response with increasing this variable (Equation 3). It is noteworthy that inhibition of growth in organisms, such as green algae, has already been evidenced against the methylparaben citoxicity by Puerta et al (2020) [9].

While the negative coefficient in the interaction between [MP] and [PP] variables was demonstrated with an antagonistic effect. The experimental combination demonstrates this effect: (*i*) concentration of [MP] = 1.2 mmol L^{-1} with [PP] = 1.2 mmol L^{-1}, 72.3% inhibition root growth; and

(*ii*) concentration of [MP] = 0.4 mmol L^{-1} with [PP] = 0.4 mmol L^{-1}, produced 57% inhibition, ie., when the Parabens concentrations are reduced, it did not evidence the increase of the toxicity efficiency. Based on the literature Nana et al (2012) [14], observed an antagonistic effect between methylparaben and propylparaben concentration against the acute toxicity of luminescent bacteria Vibrio qinghaiensis sp. Q67.

The high cytotoxicity of the mixture of methylparaben and propylparaben was observed from the mitotic index. The study was carried out comparing the negative control with the experimental combination: concentration of [MP] = 0.8 mmol L^{-1} with [PP] = 0.8 mmol L^{-1}, generating 69.3% inhibition of the root from *A. cepa*. The number of cells undergoing mitosis of the negative control was 137 cells, obtained at 13.64 ± 0.04 %MI. In contrast, the mixture of parabens decreased cell divisions for 1.48 ± 0.01 %MI with an average of 15 divisions showing a predominance of cell division in prophase. Thus, it was verified that there is a significant difference with *p* value equal to 0.0025 (value $p < 0.05$). It is worth mentioning that the percentage mitotic index was calculated with a ratio to the number of dividing cells for 1,000 cells, i.e., it is necessary to analyze a high cell division. In this context, as the MP and PP exhibited high cytotoxicity for *A. cepa*, a new experimental design (Second Factorial Design) it was carried out with a concentration (equal to 0.30 mmol L^{-1}) four times lower of the highest level (equal to 1.20 mmol L^{-1}) compared to the First Factorial Design. To evaluate the percentage of mitotic index, because the decrease in the concentration increases the cell division, due to decreased *A. cepa* cytotoxicity.

3.5 Second factorial design: Percent *A. cepa* root inhibition (% root inhibition) and percent of mitotic índex (% MI)

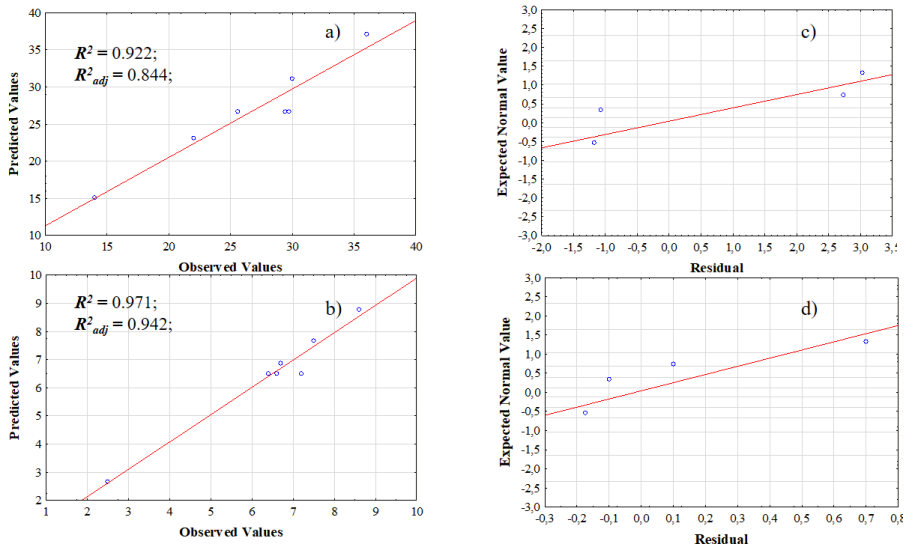

Fig. 5. Comparison of predicted and observed values for the percentage of a) % root inhibition; and b) % IM. Residual plots for the responses of the percentages of c) % root inhibition; and d) % IM.

Table 2 also collects the predicted responses generated from factorial design for the arithmetic averages of the dependent variables with 95% confidence limits. As shown Fig. 5a and 5b it was observed linear relationships presented with R^2-values between 0.922 and 0.971, as well as adjusted correlation coefficients (R^2_{adj}) between 0.844 and 0.942, for % root inhibition and % MI, respectively. For the residual analysis, it was evidenced linearity about the expected normal value (Fig. 5c and 5d) [16, 22].

Table 4 shows the analysis of variance for evaluating the influence of each independent variable, their interactions, and curvature. For a probability level of 95% high F-values and low *p*-valures (lower than 0.05) are evidence that the statistical significance for a model [24-26]. Analyzing the F-values and *P*-values (Table 4), it was not statistically significant curvature with lowed F-value equal to 2.45 and 1.65 with high *p*-value 0.258 and 0.327 for percent root inhibition and percent of mitotic índex responses, respectively. Therefore, also demonstrated that was not necessary to be applied a central composite design (CCD) model [26, 27].

3.5.1 Percent *A. cepa* root inhibition response

Fig. 6 shows the Pareto Chart that was obtained in the 0.1 to 0.3 mmol L^{-1} range (Table 2). The statistical significance ($p<0.05$) of the methylparaben concentration was evidenced. However, the relationship between [MP] (mmol L^{-1}) with [PP] (mmol L^{-1}) was not significant. Therefore, the surface plot was not generated for the percent root inhibition response.

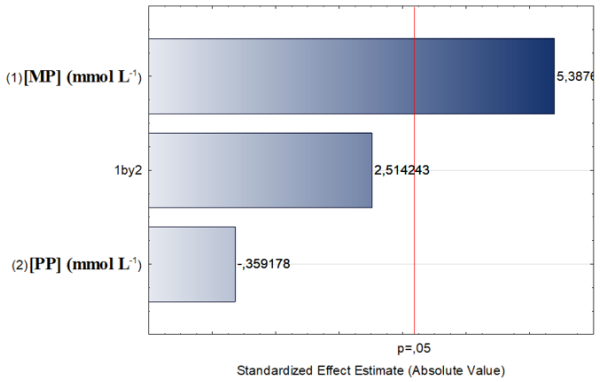

Fig. 6. Pareto chart from percent *A. cepa* root inhibition response.

Table 4. ANOVA table results for factorial design 2^3 obtained for percent root inhibition and mitotic índex responses.

Response	Factor	SS	df	MS	F-value	*p*-value
% root inhibition	Curvature	12.81	1	12.81	2.45	0.258
	[MP] (mmol L^{-1})	225.0	1	225.0	29.03	0.013
	[PP] (mmol L^{-1})	1.00	1	1.00	0.13	0.743
	[MP] *by* [PP]	49.00	1	49.00	6.32	0.087
	Error	23.25	2	7.75		
	Total SS	298.25	6			
% MI	Curvature	0.28	1	0.28	1.65	0.327
	[MP] (mmol L^{-1})	11.90	1	11.90	56.45	0.005
	[PP] (mmol L^{-1})	7.02	1	7.02	33.31	0.010
	[MP] *by* [PP]	2.40	1	2.40	11.39	0.043
	Error	0.63	2	0.21		
	Total SS	21.96	6			

SS: Sum-of-Square; df: degree of freedom; MS: Mean Square.

Equation 4 shows the value of the independent variable methylparaben concentration based on the empirical model.

$$Y_{\%inhibition} = 26.6 + 15.13X_{[MP]} \quad (4)$$

Where $X_{[MP]}$ representes the methylparaben concentration variable and $Y_{\%inhibition}$ the percentage root inhibition response for the second factorial design.

Based on the positive coefficient of the methylparaben concentration variable (Equation 4) was observed this variable increases efficiency in the root inhibition percentage. This behavior occurs in the first experimental design carried out with the statistically significant MP variable.

3.5.2 Percent of mitotic índex (% MI) response

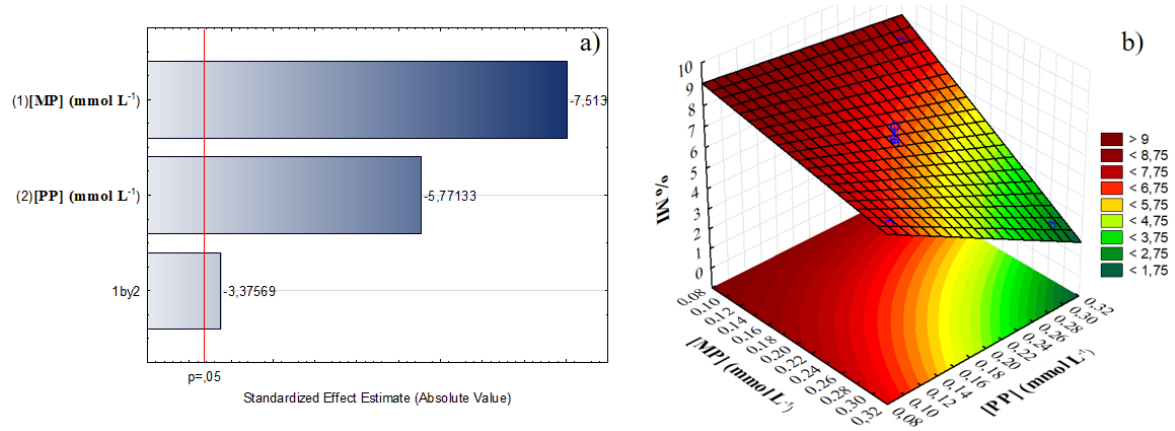

Fig. 7. a) Pareto chart; b) Response surface plot for percent mitotic índex ([MP] *vs* [PP]).

Pareto chart (Fig. 7a) shows the concentration of methylparaben, propylparaben, and the combination of variables being statistically significant ($p<0.05$). Thus, it was possible to generate a surface plot based on the dependent variable percentage of mitotic index (% MI) with the relationship: 1by2 - [MP] *vs* [PP], Fig. 7b.

The cell divisions can be carried out with an analysis of the mitotic index (% MI), which was calculated by the ratio of the number of dividing cells for 1,000 cells. Fig. 7b reveals that the highest concentration of Parabens (experiment 4: [MP] = 0.3 mmol L^{-1} and [PP] = 0.3 mmol L^{-1}) exhibited a 2.5% MI percentage with 36% inhibition of *A. cepa* root growth. While the combination with the lowest concentration in this design (experiment 1: [MP] = 0.1 mmol L^{-1} and [PP] = 0.1 mmol L^{-1}) showed 8.6 % IM and 22% root inhibition. The combination of methyl- and propylparaben exhibited high cytotoxicity because the amount of highest levels were 36% root inhibition and, still shown with a lower percentage of the mitotic index. In addition, for experiment 4, it was observed the predominance of cell division in prophase. Based on literature Medkova et al. (2023) [31] that Parabens MP and PP exhibited a potential to affect the expression of various genes playing roles in detoxification and sex hormone production for *Danio rerio* fish. Therefore, these Parabens are compounds that can be induced in cells promoting genetic and metabolic changes.

Equation 5 shows the estimated regression coefficients generate an empirical model of mitotic index percent efficiency considering variables and mutual relationships.

$$Y_{\%MI} = 6.5 - 3.45X_{[MP]} - 2.65_{[PP]} - 55X_{[MP]}X_{[PP]} \quad (5)$$

The model is favored with the lowest concentrations, due to the negative coefficients of the MP and PP concentration variables. The antagonistic effect between the variables (MP and PP concentrations) can be observed in the combinations: (*i*) concentration of [MP] = 0.1 mmol L^{-1} with [PP] = 0.1 mmol L^{-1}, presented 8.6% MI; while, (ii) concentration of [MP] = 0.3 mmol L^{-1} with [PP] = 0.3 mmol L^{-1}, obtained 2.5 %MI. Therefore, the increase in concentrations at the same time did not favor the response of the model, because the mitotic index decreases with the increase in cytotoxicity, i.e., it is a response contrary to the inhibition of the root of *A. cepa*.

4. Conclusions

It has been shown that the experimental design with a central point evaluated the interaction between the variables concentration of methylparaben and propylparaben. This interaction was based on the concentration of both Parabens with an antagonistic effect on the percentage of mortality, inhibition of root growth, and mitotic index. Acute toxicity in *A. salina* with the combination [MP] = 0.8 mmol L^{-1} and [PP] = 0.8 mmol L^{-1} caused 80% mortality. In addition, the Parabens mixture demonstrated a high cytotoxicity with *A. cepa* root inhibition that was evaluated by the percent of mitotic índex.

The comparison of two experimental design was relevant to understand the behavior of response, in this case, the percentage of root inhibition is correlated with the concentration of Parabens. In addition to understanding that contaminants can interact to increase toxicity against organisms such as *A. salina* and at the cellular level with *A. cepa*.

Acknowledgments

The authors wish to thank the Unigran Capital University Center for encouraging and financing the project. AMJ thanks the Brazilian funding agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES - Código de Financiamento 001), and Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT).

Author Contributions

Lucas de Melo da Silva: Conceptualization, Methodology, Project Administration, Supervision, Resources, Validation, Writing -Review & editing. Estela Moraes Nolasco; João Vitor dos Santos da Silva; João Vitor Vieira de Paula; Fábio Luciano Caldas da Silva: Project Execution and Writing -Review. Maicon Matos Leitão; Alessandra Silveira Antunes Araujo; Andreia de Oliveira Massulo: Conceptualization, Methodology, Resources, Writing -Review & Editing. Amilcar Machulek Jr: Conceptualization, Methodology, Writing -Review & Editing.

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How to cite this article

Nolasco, E. M.; da Silva, J. V. S.; de Paula, J. V. V.; da Silva, L. C.; Leitão, M. M.; Araújo, A. S. A.; Massulo, A. O.; Machulek Jr., A.; da Silva, L. M. *Orbital: Electron. J. Chem.* **2023**, *15*, 153. DOI: http://dx.doi.org/10.17807/orbital.v15i3.17993