Evaluation of the soluble fraction of B100 biodiesel and S500 diesel in seawater using Experimental Design

Avaliação da fração solúvel do biodiesel B100 e diesel S500 em água do mar usando planejamento experimental)

Evaluación de la fracción soluble de biodiesel B100 y diesel S500 en agua de mar mediante Diseño Experimental

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Abstract
Biodiesel is considered a clean and renewable energy source, however it is important to know the environmental impacts caused by the water-soluble fraction (WSF) of this fuel, in the event of a spill. The objective of this research was to evaluate the seawater-soluble fraction of biodiesel (B100) compared to diesel (S500). A factorial design was used to carry out the experiments and evaluate the WSF's, varying the type of fuel and the contact time between oil and water (O/W) from 1h to 24h. The mortality rate (%) of *Mysidopsis juniae* and the concentration of total hydrocarbons (TPH) were used as response variables. The results obtained demonstrate that diesel showed greater toxicity, with an average mortality rate of 98.4 % and TPH concentration of 475.8 µg/L in the water-soluble fraction. On the other hand, biodiesel did not present significant toxicity in the exposure of organisms, even after 24 hours of contact, but it can still cause risks in cases of spillage (average mortality rate of 15.0%). The empirical models obtained using the experimental design methodology were able to satisfactorily estimate the TPH concentrations and the mortality rate in the WSF, with precision above 99%, with correlation coefficients (R²) of 0.9963 and 0.9970. Pure biodiesel, due to its low lethality of organisms, biodegradable and from a renewable source, is an ecologically favorable proposal as a fuel, causing less impacts in cases of spillage in seawater.
Keywords: Water-soluble fraction; Experimental design; Biodiesel; Diesel; TPH; Acute toxicity; Mysidopsis juniae.

Resumo

O biodiesel é uma fonte energética considerada limpa e renovável, entretanto é importante conhecer os impactos ambientais causados pela fração solúvel em água (FSA) desse combustível, em caso de derramamento. O objetivo desta pesquisa foi avaliar a fração solúvel em água do mar do biodiesel (B100), comparando com o diesel (S500). Utilizou-se um planejamento fatorial para execução dos experimentos e avaliação das FSA's, variando o tipo de combustível e o tempo de contato entre óleo e água (O/A) de 1h a 24h. Foi utilizado como variáveis de resposta a taxa de mortalidade (%) dos *Mysidopsis juniae* e a concentração de hidrocarbonetos totais (TPH). Os resultados obtidos demonstram que o diesel apresentou maior toxicidade, com uma taxa de mortalidade média de 98.4 % e uma concentração de TPH total de 475.8 µg/L na fração solúvel em água. Já o biodiesel não apresentou toxicidade significativa na exposição dos organismos, mesmo após 24h de contato, mas ainda pode causar riscos em casos de derramamento (taxa de mortalidade média de 15.0 %). Os modelos obtidos utilizando a metodologia de planejamento experimental foram capazes de estimar satisfatoriamente as concentrações de TPH e a taxa de mortalidade dos M. juniae na FSA, com precisão superior a 99 %, com coeficientes de correlação (R²) de 0.9963 e 0.9970. O biodiesel puro por apresentar baixa letalidade dos organismos, biodegradável e de fonte renovável é uma proposta ecologicamente favorável como combustível, causando menores impactos em casos de derramamento em água.
Palavras-chave: Fração solúvel em água; Planejamento experimental; Biodiesel; Diesel; TPH; Toxicidade aguda; Mysidopsis juniae.

Resumen

El biodiesel es considerado una fuente de energía limpia y renovable, sin embargo es importante conocer los impactos ambientales que ocasiona la fracción hidrosoluble (FHS) de este combustible, en caso de derrame. El objetivo de esta investigación fue evaluar la fracción soluble en agua de mar del biodiesel (B100), comparándolo con el diesel (S500). Para realizar los experimentos y evaluar los FSA's se utilizó un diseño factorial, variando el tipo de combustible y el tiempo de contacto entre aceite y agua (O/W) de 1h a 24h. Se utilizaron como variables de respuesta la tasa de mortalidad (%) de *Mysidopsis juniae* y la concentración de hidrocarburos totales (HTP). Los resultados obtenidos demuestran que el diésel presentó mayor toxicidad, con una tasa de mortalidad promedio de 98,4 % y una concentración total de HTP de 475,8 µg/L en la fracción hidrosoluble. Por otro lado, el biodiesel no presentó toxicidad significativa en la exposición de organismos, incluso después de 24 horas de contacto, pero aún puede causar riesgos en casos de derrame (tasa de mortalidad promedio de 15,0%). Los modelos obtenidos mediante la metodología de diseño experimental lograron estimar satisfactoriamente las concentraciones de HTP y la tasa de mortalidad de *M. juniae* en la FHS, con precisión superior al 99%, con coeficientes de correlación (R²) de 0,9963 y 0,9970. El biodiesel puro por presentar baja letalidad de los organismos, biodegradable y de fuente renovable es una propuesta ecológicamente favorable como combustible, provocando menores impactos en casos de derrame en agua.
Palabras clave: Fracción hidrosoluble; Diseño experimental; Biodiesel; Diésel; TPH; Toxicidad aguda; Mysidopsis juniae.

1. Introduction

Annually, an increase in the consumption of fuels derived from oil is observed, in which the biggest problem is the environmental impacts that they can cause in cases of spillage and leakage in the activities of exploration, production, refining and transport of oil and its derivatives (Anjos et al. 2020; Hilário et al. 2019; Saeed et al. 2013). Diesel oil is the main fuel used in large and small fishing vessels, as well as in recreational boats and even in road transport, making it one of the most targeted petroleum products and responsible for the increase in the release of compounds harmful in the marine environment (ANP, 2021).

During a diesel oil spill or leak event in the marine environment, it spreads over the surface layer as a film a few millimeters thick, and volatile constituents evaporate in estimates of 30 to 40% of the total volume. There are also dissolution, biodegradation and emulsification processes, with the water-soluble fraction (WSF) characterized as a toxic and complex mixture of hydrocarbons (Khan *et al*., 2012). Therefore, the characterization of the adverse effects produced by such a fraction is relevant for an adequate estimation of the impacts of these spills.

A spill can also include substantial changes in oil viscosity, density and interfacial tension, producing more dangerous and water-soluble hydrocarbon fractions in the marine environment (Paul et al., 2013; Jung et al., 2017). These contaminants, while present in the environment, can promote a continuous contamination of polycyclic aromatic hydrocarbons (PAH) and total petroleum hydrocarbons (TPH), being able to generate problems for several years and even decades (Anjos et al., 2020;

Chang et al., 2014; Rengasamy et. al., 2011). Diesel oil can cause serious disturbances in the ecosystem, as its water-soluble fractions may become available to the marine biota. This bioavailability may occur due to the incrustation of the contaminant in the sediment and the solubility of hydrocarbons in water (Nogueira et al., 2011a,b). In addition, oil photolysis produces active free radicals that can react with hydrocarbons and release oxidized organic compounds into ecosystems. Such oxidized intermediate compounds are more toxic to sea biota due to high solubility and reactivity in water compared to other hydrocarbons (Zengel et al., 2016). However, biodiesel is a biodegradable fuel of plant or animal origin and it is considered less toxic than diesel, which has advantages over commercial diesel oil, such as: having a higher cetane number and low sulfur content, without aromatics and containing 10-11% oxygen by weight.

As interest in biodiesel grows, its fate in aquatic environments is an area of relevance, as fuel spills are a major source of contamination for many ecosystems. It is important to examine the biodegradability, bioavailability to organisms and toxicity in case of spillage, and compare it with fossil fuels.

The toxicity of WSF obtained from diesel and other fuels was studied for the microalgae *Dunaliella tertiolecta*, which was exposed to WST from fuel oil/diesel and it exhibited changes in growth rate and pigments, phenols, lipids and proteins concentrations (Salinas-Whittaker et al., 2010). Nogueira et al. (2011a) studied the toxic effects of oil, diesel and gasoline WSF's on larvae of the species Odontesthes argentinensis, analyzing various damages in gills, pseudo-branches and esophagus. The WSF toxicity of diesel fuel was also determined for the sea urchin *E. lucunter*, with effects on fertilization and development of pluteus larvae associated with the presence of BTEX (benzene, toluene, ethylbenzene and xylene) and PAH's (Pereira et al., 2018). Santana et. al. (2021) evaluated the diesel and bunker toxicity, analyzing total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAH's), in addition to the acute and chronic toxicity of the copepod *Nitokra sp*, the embryo-larval of the mussel Perna perna and the sea urchin *Lytechinus variegatus*. In general, WSF from diesel was more toxic to the organisms tested, but WSF from bunker was more toxic to L. variegatus embryos.

The WSF toxicity of biodiesel from different sources, such as castor oil (CO), palm oil (PO) and waste cooking oil (WCO), was studied for the microalgae *Tetraselmis chuii* and the sea urchin larvae *Echinometro lucunter*. The biodiesel toxicity levels analyzed from the WSF showed the highest toxicity for CO, followed by WCO and PO (Leite et al., 2011).

Experimental design is a tool that consists of applying statistical analysis with the aim of optimizing, modeling and experimental prediction of a studied system. It also defines the effect that independent variables have on a response of interest. This methodology is capable of reducing the time and cost of the experimental stage (Albani et al., 2020). This tool was used by Anjos et al. (2021a,b) to predict BTEX contamination in the WSF of crude oil, simulating a spill, obtaining empirical models capable of predicting these contaminants in water above 99% reliability. For an experiment to present reliability and precision in its results, there is a need for its proper planning (Anjos et al., 2021a,b; Dal'Col et al., 2020).

This work aims to evaluate the soluble fraction of diesel oil (S500) and biodiesel (B100) in sea water, using factorial design, varying the type of fuel and the contact time between oil and water. And as dependent variables, the mortality rate (%) through the acute bioassay with *Mysidopsis juniae* and the concentration of total hydrocarbons (TPH) in the soluble fraction.

2. Methodology

2.1 Materials

Diesel fuel (S500) and B100 biodiesel were provided by the Fuel and Lubricants Laboratory of the Federal University of Rio Grande do Norte (UFRN), Brazil. Seawater for the tests was acquired at the Aquatic Ecotoxicology Laboratory of the Department of Oceanography and Limnology at the Federal University of Rio Grande do Norte, Brazil. The HPLC grade methanol solvent from JT-Baker, Mexico; Sulfuric Acid 98% from Merck, Germany; Dichloromethane solvent from Supelco,

Ethyl Acetate solvent, MS gas chromatography grade from Supelco, Germany and Supelco C-18 Supelclean-SPE Tube Cartridge, Germany.

2.2 Preparation of samples of the soluble fraction of oil in water

The preparation of the soluble fraction of the oil in water for the experiments was carried out according to Anjos et al. (2021a), on a laboratory scale, with minor modifications. Briefly, in a glass flask with 2 liters of seawater (salinity was adjusted in all experiments to 34 ± 1%) was slowly added 80 mL of the oils (B100 or S500), forming a 5 mm film. The experiment was carried out at room temperature (25 °C ± 2 °C), without agitation (static). At the end of the experiments, the WSF samples were collected using a peristaltic pump BT 100S (Lead Fluid, Hebei, China) with a low flow rate (50 mL/min) and, finally, transferred to 1000 mL amber glass flasks. Figure 1 shows the preparation of WSF diesel and biodiesel in seawater.

Figure 1 - Preparation of the soluble fraction of diesel (S500) and biodiesel (B100) in seawater. (a) Addition of S500 diesel to seawater and (b) collection of the soluble fraction of B100 biodiesel with the aid of a peristaltic pump.

Source: Authors (2022).

In order to evaluate the TPH concentration in seawater and the WSF toxicity of diesel and biodiesel, a 2^2 factorial experimental design was carried out. The oil type and the contact time between oil and seawater were defined as independent variables, studying them at two levels, coded as -1 and +1 (Table 1), and, as dependent variables, the TPH concentration (ug/L) and the *Mysidopsis juniae* mortality rate (%).

Table 1 - Fatores e níveis utilizados na matriz experimental.

Factors	Symbol	Level	
		(-1)	(1)
Oil	X_1	B100	S50
Contact time O/W	X_2	1	24

Source: Authors (2021).

Assays were performed in duplicates with a total of 8 assays. Data on TPH concentration (ug/L) and *Mysidopsis juniae* mortality rate (%) were subjected to regression analysis, using the computer program Statistica version 7.0 in order to obtain the main effects of the studied variables, as well as the analysis of variance (ANOVA) to verify the empirical models

(linear) obtained from the experimental design. The coefficient of determination (R^2) and the F test were used as parameters for evaluating the models (Barros Neto et al., 2010).

2.3 TPH analysis by GC/FID

The WSF samples were analyzed based on the USEPA 8015C method, using the gas chromatograph with a flame ionization detector (FID), GC 2010 from Shimadzu (Kyoto, Japan). For chromatographic separation, a ZB-5MSi column (Phenomenex, California, USA) was used. The carrier gas used was nitrogen (99.999% purity, Linde Gases Ltda, Brazil) with a constant flow of 2.5 mL/min. The temperature programming was: 40 °C maintained for 5 min, following a heating ramp of 100 °C, at a rate of 10 °C/min. The temperature was then raised to 330 °C at a rate of 12 °C/min, remaining at this temperature for 12 min. The injector temperature was 220 °C. The calibration standard for identifying retention times for n-alkanes C8 to C40 was purchased from AccuStandard (DRH-008S-R2). A microliter of the sample diluted to a final volume of 1 mL with n-hexane was injected.

Samples were initially prepared using the extraction method based on USEPA 3535a. The extraction started with the addition of sulfuric acid until pH < 2 to the samples and 10 mL of methanol, followed by common filtration of 1000 mL, using a volumetric flask, funnel and filter paper. The filtered samples were taken to the extractor equipment, Manifold brand SUPELCO, Sigma-Aldrich. The C-18 (octadecylsilane) cartridges of the SUPELCO brand, Sigma-Aldrich, were washed with 5.0 ml of ethyl acetate, followed by 5 ml of dichloromethane, conditioned with 10 ml of methanol and a further 10 ml of ultrapure water. Then, the samples were passed through the cartridges, the analyte being retained by affinity between the organic compounds present in the samples and the adsorbent present inside the cartridges. The organic phase was eluted with 5 ml of ethyl acetate and 5 ml of dichloromethane. The cartridges were dried with nitrogen until approximately 2.5 mL of extract was obtained. The extract was transferred to a vial and taken to a chromatograph for quantification of TPH.

2.4 Acute toxicity test

The marine microcrustacea *Mysidopsis juniae* used for the tests of acute toxicity of the WSF's were cultivated in the Laboratory of Aquatic Ecotoxicology ECOTOXLAB of the Nucleus of Primary Processing and Reuse of Produced Water and Waste at UFRN, Brazil. The culture was maintained under controlled conditions in aquariums (8 L) with filtered natural seawater (0.5 μm filter) and proportion ratio of 20 males to 60 females. The abiotic conditions were as follows: seawater salinity at 35 ± 1%; 12h light / 12h dark photoperiod; temperature of 25 ± 2 °C; constant aeration. The test organisms *Mysidopsis juniae* were fed with the nauplii of the microcrustacea *Artemia sp*. enriched with cod liver oil and fish oil (age: 48h) daily.

Acute toxicity tests were based on NBR 15308 (ABNT, 2017), in which ten juveniles of *M. juniae* (1–8 days old) were exposed in triplicate for 96 hours to the water-soluble fraction of oils, in addition to a control negative filtered seawater. Test organisms were fed daily with Artemia sp. The pH, salinity and dissolved oxygen concentration (DO) were measured at the beginning and at the end of each experiment, using a Hanna Multiparameter - HI9829 (Hanna Instruments, Limena, Italy). The test validation criteria were based on control survival, which must be greater than 90%, and sensitivity to zinc as a reference substance (ABNT, 2017).

At the end of each test (96h), the organisms were considered dead when immobilized after gentle mechanical stimulation, and the percentage of survival of the organisms at the end of each test was calculated.

3. Results and Discussion

Although the use and production of biodiesel has been widely encouraged as a renewable alternative to petroleum diesel, the potential toxicity of this new fuel in the event of a spill is still poorly understood (Brandão et al., 2021). This work sought to evaluate the toxicity and total hydrocarbons in the WSF of pure biodiesel (B100) and commercial diesel (S500) through experimental design with a 2^2 factorial matrix, randomly executed in duplicate. The experimental matrix and the results are presented in Table 2.

Table 2 - The experimental matrix 2^2.

Experiment	Oil	Contact time O/W (h)	TPH (µg/L)	Mortality rate (%)
1	-1 (B100)	-1 (1)	179.9	13.3
2	1 (S500)	-1 (1)	229.0	10.0
3	-1 (B100)	1 (24)	231.4	10.0
4	1 (S500)	1 (24)	483.0	96.7
5	-1 (B100)	-1 (1)	187.0	16.7
6	1 (S500)	-1 (1)	183.7	10.0
7	-1 (B100)	1 (24)	236.2	16.7
8	1 (S500)	1 (24)	468.6	100.0

Source: Authors (2022).

According to Table 2, when using the O/W contact time of 1h and oil type variation from B100 to S500, the average concentration of TPH goes from 183.5 µg/L to 206.4 µg/L, with an increase of 12.5%, however, the *Mysidopsis juniae* mortality rate for WSF is slightly higher for the B100 sample than for the S500. This may have possibly occurred due to the presence of unknown elements in the biodiesel composition, such as methanol or ethanol impurities from reverse transesterification reactions, or even reactive compounds from the auto-oxidation of fatty acids present in biodiesel (Cavalcante et al., 2014; da Cruz et al., 2012; Nogueira and Almeida, 2012; Nogueira et al., 2011a,b; Leite et al., 2011).

With 24 hours of O/W contact and still varying the type of oil B100 to S500, it is observed that the average concentration of TPH in µg/L increases significantly by 50%, that causes greater bioavailability of hydrocarbons in the WSF, possibly being more toxic to organisms, which was confirmed by the increase in the mortality rate from 13.4 % ± 4.7 for the B100 to 98.4 % ± 2.3 for the WSF of the S500 diesel.

In Table 2, it is also possible to observe that, when fixing the B100 oil and varying the time from 1 to 24h, there was a small increase of 50 µg/L in the TPH concentration of the water-soluble fraction. For S500, there was a significant increase in TPH concentration of 269.4 µg/L and an increase of 88.4% in the mortality rate of *Mysidopsis juniae*. The soluble fraction of diesel oil contains many polycyclic aromatic hydrocarbons (PAH) that can cause damage to the liver and gills of fish and oxidative stress, as studied by Simonato et al. (2008) and Nogueira et al. (2011a), and even the water-insoluble fractions can cover the surface of organisms, causing acute toxicity and mortality.

The soluble fraction of the B100 oil did not show a significant variation in the mortality rate with the O/W contact times from 1 to 24h, in which an average of 4.5 and 4 organisms died in the acute tests (96 h), respectively, showing that in the

first 24 hours there is no acute toxicity for the organisms under study in cases of spillage in the marine environment. Figure 2 shows the relationship between the concentration of TPH and the Mortality rate of soluble fractions according to the experimental matrix, in which it is possible to observe, with 24h of O/W contact and using S500 diesel oil, the highest average TPH concentration in µg/L, as well as the mortality rate, that is, more toxic to *Mysidopsis juniae* organisms (Experiments 4 and 8). It is also observed that the mortality rate of B100 in Experiments 1 and 3 and Experiments 5 and 7 remained constant. In the standard NBR 15308:2017 of the Brazilian Association of Technical Standards (ABNT, 2017), it is considered as a criterion for validation of acute toxicity tests the survival of the control group to be at least 90%, that is, a mortality rate of up to 10% of organisms. As observed for the vast majority of trials, mortality rates ranged between 10% and 16.7%, with low toxicity. On the other hand, commercial diesel with 24 hours of contact with seawater had a high mortality rate, ranging from 96.7 to 100% (Table 2), with WSF being the most toxic and at risk in cases of spillage.

Figure 2 - Relationship between TPH concentration and *Mysidopsis juniae* mortality rate.
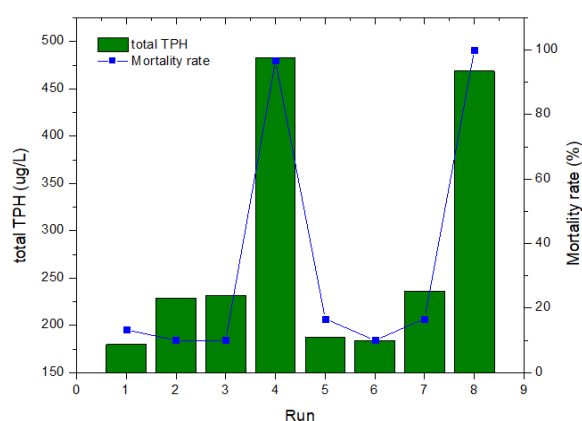
Source: Authors (2022).

To assess the statistical significance of the empirical models, analysis of variance (ANOVA) was performed at a confidence level of 95%. According to the analysis of variance performed, it was found that the models are significant with a correlation coefficient of R^2 of 0.9963, R-adj of 0.9935 and $p < 0.05$ and R^2 of 0.9970, R-adj of 0.9947 and $p < 0.05$ for TPH and mortality rate, respectively (Tables 3-4). The $F_{calculated}/F_{tabulated}$ ratio for the TPH was 39.44 and the *M. juniae* mortality rate was 47.80. The F test confirms that $F_{calculated}$ was greater than five times the value of $F_{tabulated}$, and can be used for predictive purposes (Barros Neto *et al*., 2010).

Table 3 - Analysis of Variance of TPH.

Source	Sum of squares	DF1	Mean squares	F	p
(1)Oil	38147.4	1	38147.36	383.6888	0.000040
(2)Contact time (h)	47566.1	1	47566.07	478.4229	0.000026
1 by 2	21581.1	1	21581.07	217.0639	0.000124
Regression	107294.508		3	Significant and predictive	
Resíduals	397.691		4		
Total	107692.198		7		
R-sqr = 0.9963	R-adj = 0.9935		Fcalc = 435.93	Fcalc/Ftab = 47.80	

1 - DF – Degree of freedom; R-sqr - Coeficiente de correlação; R-sqr - R ajustado; Ftab = 9.12. Source: Authors (2022).

**Table 4 -** Analysis of Variance of *Mysidopsis juniae* mortality rate.

Source	Sum of squares	DF1	Mean squares	F	p
(1)Oil	38147,4	1	38147,36	383,6888	0,000040
(2)Contact time (h)	47566,1	1	47566,07	478,4229	0,000026
1 by 2	21581,1	1	21581,07	217,0639	0,000124
Regression	107294,508		3	Significant and predictive	
Resíduals	397,691		4		
Total	107692,198		7		
R-sqr = 0.9970	R-adj = 0.9947		F_{calc} = 359.73	F_{calc}/F_{tab} = 39.44	

1 - DF – Degree of freedom; R-sqr - Coeficiente de correlação; R-sqr - R ajustado; Ftab = 9.12. Source: Authors (2022).

Considering that the models were well adjusted (significant and predictive), the effects of the variables and the interactions between them on the TPH concentration and the mortality rate of *M. juniae* in the WSF's were evaluated based on Pareto diagrams (Figure 3), at the 95% confidence level.

Figure 3 - Pareto Graph (a) TPH, (b) *Mysidopsis juniae* mortality rate, effects (c) of the oil variable and (d) contact time on TPH concentration and mortality rate in WSF's.
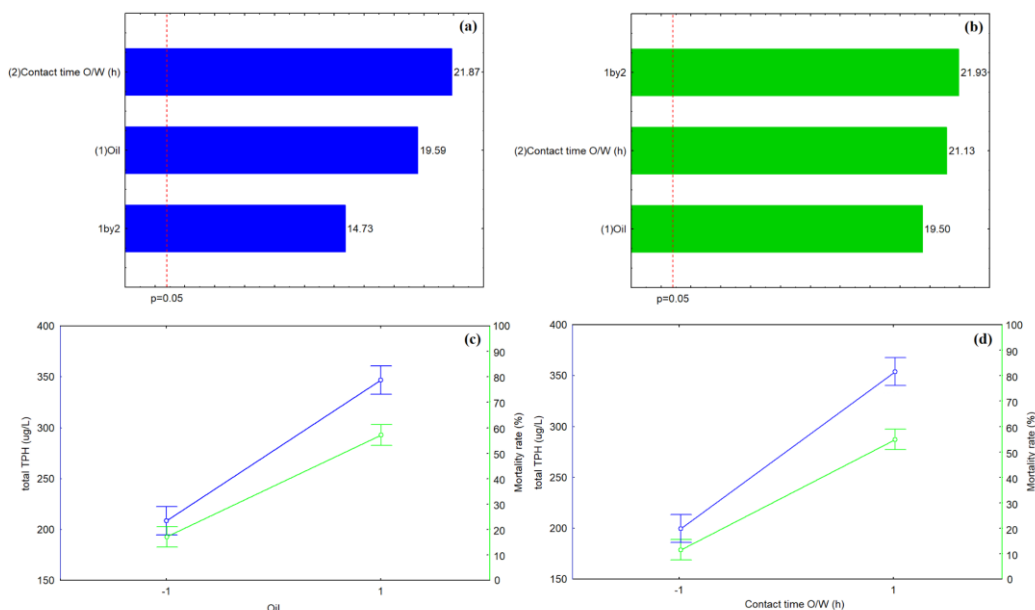
Source: Authors (2022).

For the TPH concentration in the soluble fraction of oils in seawater, using the Pareto graph (Figure 3a), it appears that the contact time is the main factor that affects the increase in the TPH concentration. Anjos et al. (2021a,b) observed an increase in the concentration of BTEX (benzene, toluene, ethylbenzene and xylenes) in seawater with an increase in the contact time for a light and medium oil (°API) in the WSF. It can be seen in Figure 3a-b that, when changing from level -1 (1h) to level +1 (24h), there is an increase in the standardized effect (absolute value) of 21.87 and 21.13 for TPH and mortality rate, respectively. For the mortality rate of *M. juniae* in WSF, the interaction between the type of oil and the contact time had the greatest significant effect, that is, when we changed the oil from B100 to S500 (Figure c), and increased time oil/water contact from 1h to 24h (Figure d), we increase the mortality rate, consequently the toxicity of WSF. These data corroborate that fossil fuel (diesel S500) has greater toxicity compared to biodiesel in cases of spillage in water (Khan et al. 2012). In this work, when comparing biodiesel and diesel, it was found that the WSF of this B100 presented lower levels of toxicity when compared to the WSF of S500. These results are similar to the work of Müller et al (2019), which analyzed WSF of biodiesel, diesel and a binary mixture of 5%, in which using the aquatic microcrustacea Daphnia magna and the marine bacterium *Aliivibrio fischeri* as test organisms, highlighted biodiesel as an acute toxic level fuel and excluding the presence of chronic toxicity.

A toxicity rating can be performed based on the fatality rate, as the higher the fatality percentage, the more toxic the fuel. Of the samples of the soluble fractions tested, diesel was the one with the highest toxicity (98.4%), while B100 was the least toxic (15.0%). Based on these results, it can be concluded that biodiesel is considerably less toxic to marine organisms than fossil diesel. Although biodiesel (B100) is less toxic than S500, the results showed some risk to *M. juniae* with mortality ranging between 13.4 - 15.0% in the spill time between 1 and 24h. Consequently, there would still be potential impacts to aquatic organisms if B100 were accidentally spilled during transport, storage or use.

In future works, it is important not only to perform acute toxicity tests with *Mysidopsis juniae*, observing the lethality effect to calculate the mortality rate (%), but also to carry out tests that aim to determine the mean Lethal Concentration (LC50) or the Average Effective (EC50), that is, the concentration of the toxic agent that causes mortality or immobility,

respectively, at 50% of the test organisms, in addition to varying the biodiesel contents in diesel (Khan *et al*. 2012) and analyzing the WSF's.

With the data obtained in the experimental design and in the regression analysis, it was also possible to obtain empirical models capable of predicting the TPH concentration and the mortality rate of *M. juniae* in the fractions soluble in seawater, depending on the type of oil (B100 and S500) and the oil/water contact time. These second-order polynomial equations (Equation 1-2) explain the relationship of each response to significant factors and interactions, with coded values for the input variables found by statistical modeling.

$$TPH \ (\mu g/L) = 287.47 + 69.05X_1 + 77.11X_2 + 51.94X_1X_2 \qquad (1)$$

$$Rate \ mortality \ (\%) = 34.18 + 20.00X_1 + 21.68X_2 + 22.50X_1X_2 \qquad (2)$$

Where X_1 and X_2 correspond to the oil type and oil/water contact time, respectively.

The model adjustments can be seen in Figure 3a-b through the relationship of experimental and predicted values for TPH concentration and mortality rate. They can be used as a tool to predict the concentration of TPH and mortality rate in cases of spillage in seawater (Anjos et al. 2021a,b) with accuracy greater than 99%, within the ranges studied in this work (Table 3-4), as well as predicting possible deleterious effects on marine environments from diesel (S500) or biodiesel (B100) spills.

Figure 4 - Observed values and predicted values by the models: (a) TPH and (b) Mortality rate.
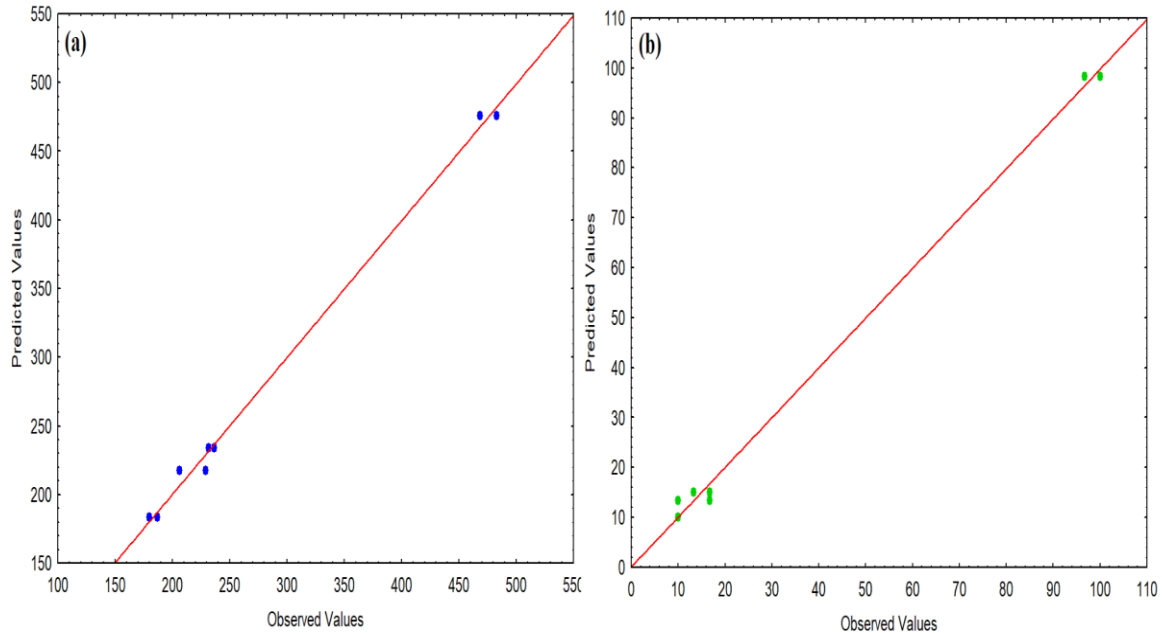
Source: Authors (2022).

4. Conclusion

The present study proved that the soluble fraction of diesel had a higher mortality rate for *Mysidopsis juniae*, and this effect was dependent on the contact time between oil and seawater, and on the type of fuel spilled. The TPH concentration in the soluble fraction of the oils increased with time from 1 to 24h, becoming more bioavailable to organisms, demonstrating the potential impact of these water-soluble compounds on marine life. Biodiesel B100, even with low acute toxicity to *M. juniae*,

shows some risk between 1 and 24 hours of contact with oil and water, requiring care during transport, storage or use. In addition, factorial planning was a valid tool in the evaluation of contamination after the spill of B100 and S500 oils in cases of spills up to 24h, presenting empirical models capable of quantitatively predicting the contamination of TPH and the mortality rate of *Mysidopsis juniae* with precision greater than 99%. As a future proposition, a study of the toxicity of the water-soluble fractions of biodiesel and diesel at different concentrations should be carried out.

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