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Líquidos geradores de espumas (LGEs) e os agentes encapsuladores (EAS) podem interferir na reprodução e no crescimento de *Daphnia similis*?

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Dissertação apresentada a Universidade Santa Cecília como parte dos requisitos para obtenção de título de mestre no Programa de Pós-Graduação em Sustentabilidade de Ecossistemas Costeiros e Marinhos, sob a orientação da Prof. Dr. Camilo Dias Seabra Pereira e coorientação da Profa. Dra. Luciane Alves Maranho.

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“Viver uma vida sem temores e sem medos é o dom que eu recebi. E, assim, começa a minha jornada."

Halle Berry (2004)

RESUMO

No combate a incêndios de classe B, os agentes de supressão de incêndio (FSA), como Líquidos Geradores de Espumas (LGEs) e agentes encapsulantes (EA) têm a finalidade de resfriar, suprimir e remover a superfície em chamas, mas vários estudos apontam para o ambiente aquático como o destino final desses compostos estáveis, bem como a liberação de compostos perfluorados em suas formas de degradação. Com características de persistência, a toxicidade dos compostos per- e polifluoroalquílicos (PFASs), especialmente os FSAs, levanta questões sobre a saúde ambiental. Neste estudo, a reprodução, maturação e comprimento corporal do microcrustáceo aquático *Daphnia similis* foram analisados através da exposição de organismos a diferentes marcas comerciais de 2 FSAs, Cold fire® Supressant Agent e Liovac®, nas seguintes concentrações: 0,000093%; 0,0001875%; 0,000375%; 0,00075%; 0,0015%, 0,0003125%; 0,000625%; 0,00125%; 0,0025%; 0,005%, respectivamente. Nossos resultados mostraram que a exposição a LGEs e EA causou efeito inibitório na reprodução de *Daphnia similis*. Além disso, houve um atraso significativo na maturação e diminuição significativa do comprimento corporal em indivíduos expostos a concentrações menores que as recomendadas pelos fabricantes. Esses resultados destacam a importância de estudos futuros com compostos perfluorados de cadeia longa e curta, por serem necessários considerar os diferentes efeitos causados por elas e, se necessário, apenas o uso de formulações que tenham menor impacto ecotoxicológico.

Palavras-Chave: Compostos perfluorados. PFAS. Microcrustáceo. Agentes supressores de incêndios. Água doce.

ABSTRACT

Could aqueous film-forming foams (AFFFs) and encapsulator agents (EAs) interferes on the reproduction and growth of *Daphnia similis*?

In firefighting of class B, fire suppression agents (FSA) such as aqueous film-forming foams (AFFF) and encapsulating agents (EA) has the purpose to cool, suppress and remove the burning surface, but several studies point to the aquatic environment as to the destination of these stable compounds, as well as the release of perfluorinated compounds in their degradation forms. As characteristics of persistence, the toxicity of the per- and polyfluoroalkyl compounds (PFASs), especially the FSAs, raises about the environmental health concerns. In this study, the reproduction, maturation, and body length of the aquatic microcrustacean *Daphnia similis* were analyzed through the exposure of organisms to different commercial brands of 2 FSAs, Cold fire® Suppressant Agent and Liovac®, in the following concentrations: 0.000093%; 0.0001875%; 0.000375%; 0.00075%; 0.0015%, (0.0003125%; 0.000625%; 0.01025%; 0.025%; 0.005%, respectively. Our results showed that exposure to AFFFs and EA caused inhibitory effect on reproduction of *Daphnia similis*. In addition, there was a significant delay in maturation and a significant decrease in the body length in individuals exposed to lower concentrations than those recommended by manufactures. These results highlight the importance of future studies with long- and short-chain-perfluorinated compounds because it is necessary to consider the different effects caused by them, and if necessary, only the use of formulations that have less ecotoxicological impact.

Keywords: Perfluorinated compounds. PFAS. Microcrustacean. Fire Suppression Agents. Freshwater.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

ABNT NBR	-	Associação Brasileira de Normas Técnicas
AFFF	-	Aqueous film- forming foams
CETESB	-	Companhia Ambiental do Estado de São Paulo
COLD FIRE®	-	Commercial name: Cold fire® suppressant agente
EA	-	Encapsulator Agents
ED	-	Endocrine Disruptors
ETHER-PFAS	-	Chlorinated polyfluorinated ether sulfonate substances
FSA	-	Fire Supression Agents
KCL	-	Potassium Chloride
LIOVAC®	-	Commercial name: Liovac® Fire
LGE-AFFF	-	Líquidos Geradores de Espumas
LOEC	-	Lowest Observed Effect Concentration
NOEC	-	No Observed Effect Concentration
OECD	-	Organisation for Economic Co-operation and Development
PFASS	-	Per- and polyfluoroalkyl substances
PFOF	-	Perfluorooctane sulfonyl fluoride
PFCAS	-	Perfluoroalkyl carboxylic acids
PFNA	-	Perfluorononanoic acid
PFOA	-	Perfluorooctanoic acids
PFOS	-	Perfluorooctane sulfonic acids
POPS	-	Persistent Organic Pollutants
UL	-	Standard for Foam Equipment and Liquid Concentrates Underwriters Laboratories
(µm)	-	Micrometer scale

SUMÁRIO

1. INTRODUCTION

Per- and polyfluoroalkyl substances (PFASs) represent a family of synthetic chemicals, obtained by several companies since the 1940s. PFAS are employed in different materials or industrial applications, such as cosmetics, food packaging, non-stick cookware, paints, medical devices, petroleum production, mining, pesticides, waterproof clothes and Fire Suppression Agents (FSA) as of Aqueous Film Forming Foams (AFFF) (Liovac® and other chemical brands) and Encapsulator Agents (EA) (Cold Fire® and other commercial brands), for firefighting control(PREVEDOUROS *et al.*, 2006; WANG *et al.*, 2017; DA SILVA *et al.*, 2019; DANIEL *et al.*, 2021).

In large-scale fires, such as flammable liquids (class B), the use of only water does not offer rapid resolution and effectiveness. The low viscosity of the water provides an increase in the flow of the flame that leads to the spread of the fire (FIGUEREDO *et al.*, 1999), since its high surface tension prevents its penetration into the burning material (DALTIN, 2011; DANIEL *et al.*, 2021).

Thus, more efficient methods are used, such as FSA (BOURGEOIS *et al.*, 2015; DANIEL *et al.*, 2021) with properties to produce a fine FSA layer that cool, suppress, and eliminate the fire on the surface (FIGUEREDO *et al.*, 1999). FSA has a high thermal and chemical stability guaranteed unique property of reducing the surface tension of molecules (GOMIS *et al.*, 2018; DANIEL *et al.*, 2021).

However, studies reported several chemical substances in the composition of these liquids, as solvents, additives, per- and polyfluoroalkyl substances (MOODY and FIELD 2000; BACKE *et al.*, 2013; DANIEL *et al.*, 2021). In the last stages of perfluorinated compounds degradation, there are present perfluorooctane sulfonic acids (PFOS) and perfluorooctanoic acids (PFOA) (MONTAGNOLLI, 2015; BARZEN-HANSON *et al.*, 2017). These compounds are considered stable due to the carbon-fluorine bond and persistent in aquatic ecosystems (BUTENHOFF *et al.*, 2006). PFOA and PFOS are described as bioaccumulative in several species of aquatic organisms, in human tissues and vegetables, which may lead to trophic biomagnification (GRANDJEAN *et al.*, 2017; MCCARTHY *et al.*, 2017; LIU *et al.*, 2018; ABERCROMBIE *et al.*, 2019; LI *et al.*, 2019; SIMONNET-LAPRADE *et al.*, 2019). The occurrence of these compounds in organisms was observed worldwide, including the poles (GIESY and KANNAN, 2001; BOISVERT *et al.*, 2019).

The compounds have toxic potential with a possible mode of action as cellular oxidative stressors in aquatic organisms (OECD, 2002; DA SILVA *et al.*, 2019; YANG *et al.*, 2019). Several studies reported an inhibitory effect on growth in algae species, representing a threat to base organisms of the food chain (BOUDREAU *et al.*, 2003; SANDERSON *et al.*, 2003; NIU *et al.*, 2019). Studies have described PFOS and PFOA as endocrine disruptors (ED) (WIELOGÓRSKA *et al.*, 2015; NAIDU *et al.*, 2016). ED can manifest its effects at any stage of this hormonal dynamic (CRAIN *et al.*, 2000) with severe changes in critical periods such as embryonic development that may cause changes in different phases in the life cycle, passing to the next generations (FILHO *et al.*, 2007; COPERCHINI *et al.*, 2017).

As a result of toxicologically worrying issues conducted by the Review Committee for Persistent Organic Pollutants (POPs), the Stockholm Convention held in 2004 decided to restrict the production, import, export, use and disposal of a list of components considered as POPs (CETESB, 2020). The Stockholm Convention Annex B classifies substances as restricted use, with the perspective to be eliminated in the future. In 2009, a new list of POPs was added including perfluorooctane sulfonic acid and its salts (PFOS), perfluorooctane sulfonyl fluoride (PFOSF) and PFOAs in Annex B (WANG *et al.*, 2009; CETESB, 2020). In 2019, perfluorooctanoic acid (PFOA) and its salts were added to Annex A to its total elimination (UNEP 2015; 2017; RIPLEY; SYNERGIES 2019).

The use of AFFFs is still on a large scale worldwide, in firefighting that occur in industries (KISHI and ARAI, 2008), airports (AWAD *et al.*, 2011), forests (BOULTON *et al.*, 2003) and military bases (BACKE *et al.*, 2013). The fire at the Petrochemical Terminal, located in the municipality of Santos, on the coast of the State of São Paulo, Brazil, the use of> 61,000l of FSAs has been reported, lasting approximately 10 days (DA SILVA et al., 2019), considered the biggest chemical disaster ever recorded in Brazil, since the Campos basin, located in Rio de Janeiro in 2011 (FOLHA DE SÃO PAULO, 2011, 2015).

The genus *Daphnia* is a well-studied aquatic microcrustacean group with standard acute and chronic ecotoxicological tests for being easily cultivated in the laboratory, for having a short life cycle, being sensitive to various aquatic contaminants and reproducing asexually by parthenogenesis, which originates genetically identical descendants, and constantly maintains the attainment of sensitive test organisms (CHEN *et al.*, 2019; PAGLIARINI *et al.*, 2019; ROSA *et al.*, 2019). The test species *Daphnia similis* was selected because it has a large distribution in freshwater ecosystems, acts as a primary consumer, and is a major food source for higher trophic levels in various food chains. As well as the organisms were recommended and validated by several guidelines OECD 211 (2012) and NBR 12713 (2016). To this end, neonates (*D. similis*) were exposed to the selected AFFFs in chronic toxicity tests. The reproduction, the body length, and the time to the first brood of the organism in different concentrations of FSAs were noted.

2. MATERIALS AND METHODS

2.1 TEST ORGANISM

D. similis female neonates (< 24 h old) used in this study were obtained from permanent cultures of *Caetano Belliboni ecotoxicology laboratory*. All the physical-chemical parameters were according to the standard *D. similis* protocol ABNT NBR 12713 (2016), (values to pH 7.0 to 7.6 and water hardness between 40 and 48 mg $CaCO_3$/L). The sensitivity of the organisms used in the culture was evaluated using the KCl test, and presented EC50= 310.27 mg/L. This result is within the acceptable sensitivity range for the species (239.28 mg/L to 381.24 mg/L), according to the Control Letter of the Laboratory of Ecotoxicology at UNISANTA.set by the NBR 12713 (2016).

2.2 TOXICITY TEST

Commercial formulations of Fire Suppression Agents: Cold Fire® (Encapsulator Agent) and Liovac® (Aqueous film-forming foams), both in formulations diluted to 10% (v/v) in ultrapure water for better dissolution, were used. The concentrations were based on the acute test performed by Da Silva *et al.* (2019).

For each treatment, ten glass beakers (20mL) containing an individually neonate (< 24h old) were used and exposed to one of the following treatments: Control (water), 0,000093%; 0,0001875%; 0,000375%; 0,00075%; 0,0015% of Cold fire and 0,0003125%; 0,000625%; 0,00125%; 0,025%; 0,005% of Liovac®. The chronic test exposure was performed on the first brood neonates of *Daphnia* following OECD 211 (2012) guidelines lasting 21 days. The neonates were fed daily with green algae *Raphidocelis subcapitata* and kept in a germination chamber in a photoperiod of 16: 8h light: dark, with luminous intensity from 500 to 1000 lux and with a temperature around 20 ± 2ºC. Every two days, the organisms were moved into clean glass beakers using a Pasteur pipette, to minimize as much as possible any stress generated and maintain the test concentrations. All the neonates obtained were noted as well as the mortality if it occurred.

2.3 BODY LENGTH

The ZEISS Stemi 2000-C binocular stereo microscope was used, calibrated at 2.5 magnification to measure the body length of the surviving organisms. Each organism was photographed, and its body length was measured on a micrometer scale (μm), using the same Stereomicroscope, with the aid of an integrated camera (AxioCam ICc 3).

2.4 STATISTICAL ANALYSES

For all analyzes significance level of ($p \leq 0.05$) was adopted. The data obtained by monitoring adult *Daphnias* as well as the average reproduction of each treatment, over 21 days, were treated by the Grubbs test to remove outliers, and then were subjected to the normality and homoscedasticity tests by the Shapiro-Wilk and Bartlett, respectively. The results obtained by the analysis of reproduction and body length of neonates exposed to Cold Fire® and Liovac® (body length only) were compared with the respective controls by ANOVA analysis of variance “one way”, followed by Bonferroni's post hoc test ($p < 0.05$). The reproduction results of the organisms submitted to Liovac® concentrations were submitted to the Wilcoxon test. To verify differences in the time of the first reproduction of the *Daphnia* between the concentrations of the compounds, an ANOVA “one way” was used followed by the Tukey test posteriori ($p < 0.05$). Statistical analyzes were performed using GraphPad Prism 5 Software and Toxstat 3.5 (WEST and GULLEY, 1996).

3. RESULTS

3.1 EFFECTS ON REPRODUCTION AND TIME TO THE FIRST BROOD

Control survival was 100%, well above the limits set by the OECD (2012) guideline (>80%). The average number of neonates per control female after 21 days was 51.4 neonates per daphnid. The Lowest Observed Effect Concentration (LOEC) for Cold Fire® was observed in the 0.0001875% dilution with decreased reproduction ($p < 0.05$) when compared to the control (Figure 1), and delay for time to the first brood of neonates ($p < 0.05$), (Figure 2). The No Observed Effect Concentration (NOEC) for the same contaminant can be attributed to a concentration less than 0.000093% (Figure 1). The LOEC for Liovac® was noted at the concentration of 0.005% with the significant reproductive decline ($p< 0.05$), (Figure 3) and significant delay for time to the first brood of neonates ($p < 0.05$) (Figure 4), when compared to the control. NOEC was observed at the concentration 0.0025% because the neonates obtained during the monitoring were not significantly different ($p < 0.05$) from the control (Figure 3).

3.2 EFFECT ON BODY LENGTH

For Cold Fire®, a significant decrease ($p < 0.05$) in the body length of surviving *daphnia* exposed to all concentrations was observed, when compared to the control (Figure 5). For Liovac®, no significant difference was observed ($p < 0.05$) in the length of the organisms exposed to the contaminant, when compared to the control (Figure 6).

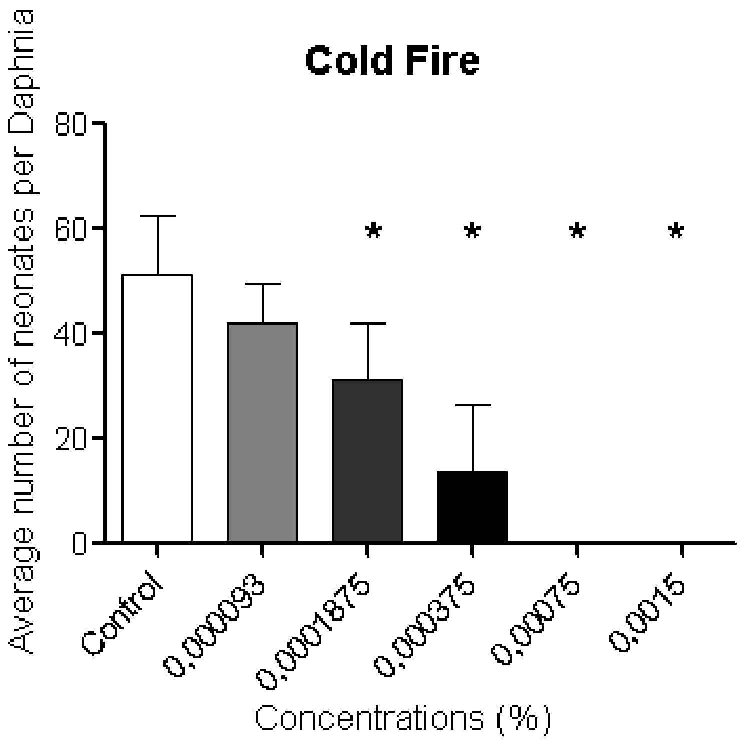

Figure 1. Average reproduction of *Daphnia similis* exposed to concentrations of Cold Fire®.
Asterisk (*) = significantly different from the control (p <0.05).
Source: Elaborated by the author.

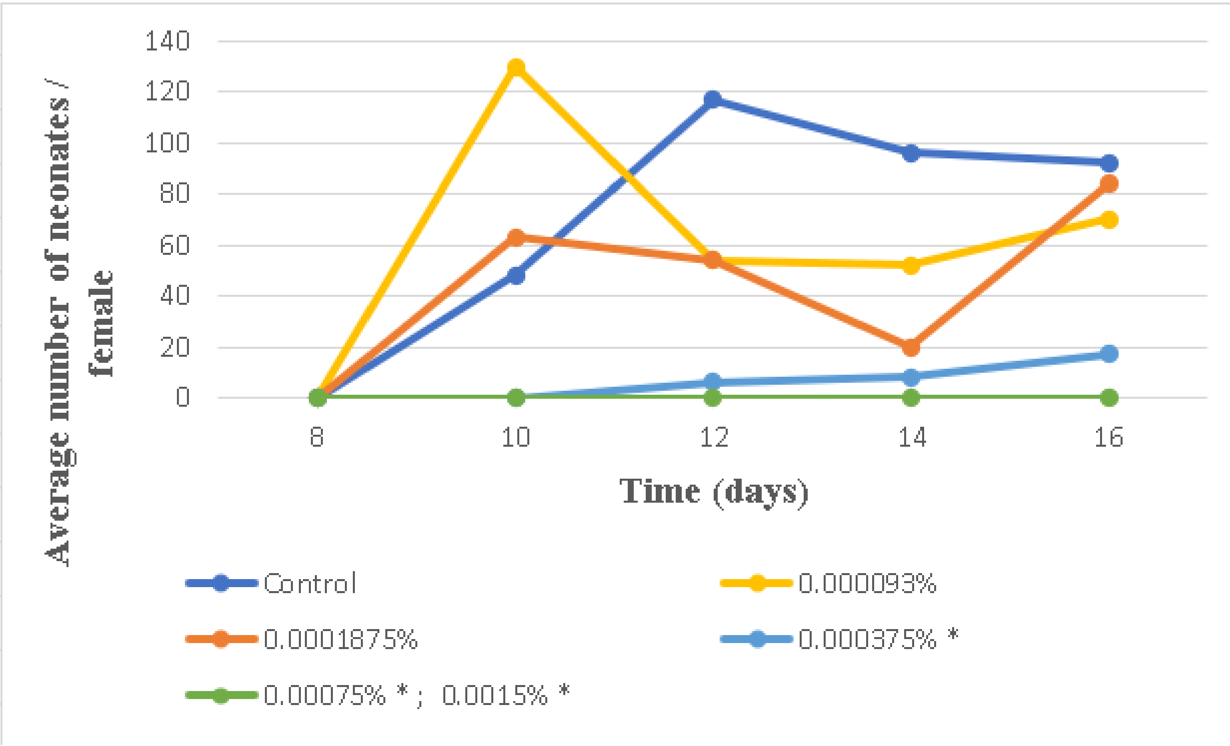

Figure 2. Time for the first brood (days) over 21 days after exposure to Cold Fire. Asterisk (*) = significantly different from the control ($p < 0.05$).
Source: Elaborated by the author.

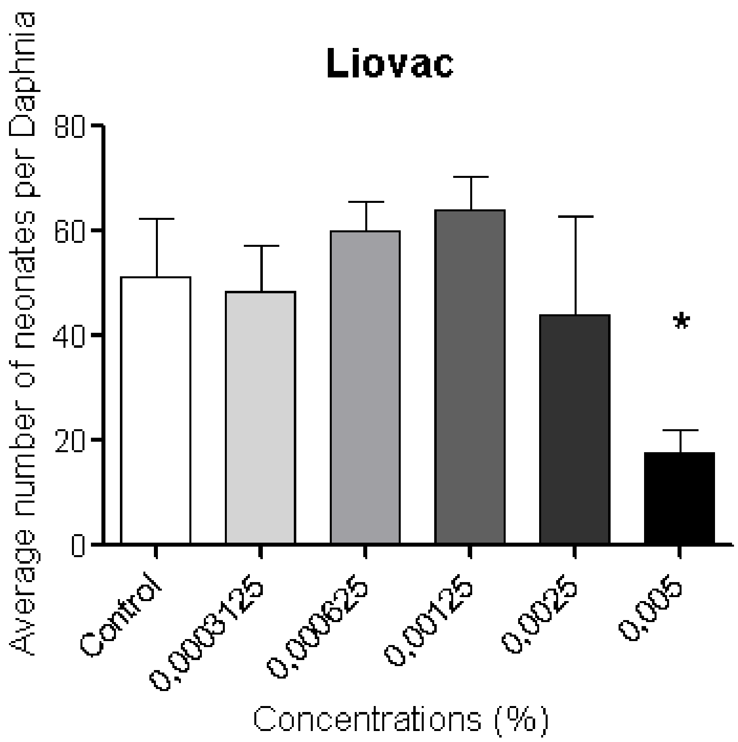

Figure 3. Average reproduction of *Daphnia similis* exposed to concentrations of Liovac®. Asterisk (*) = significantly different from the control ($p < 0.05$).
Source: Elaborated by the author.

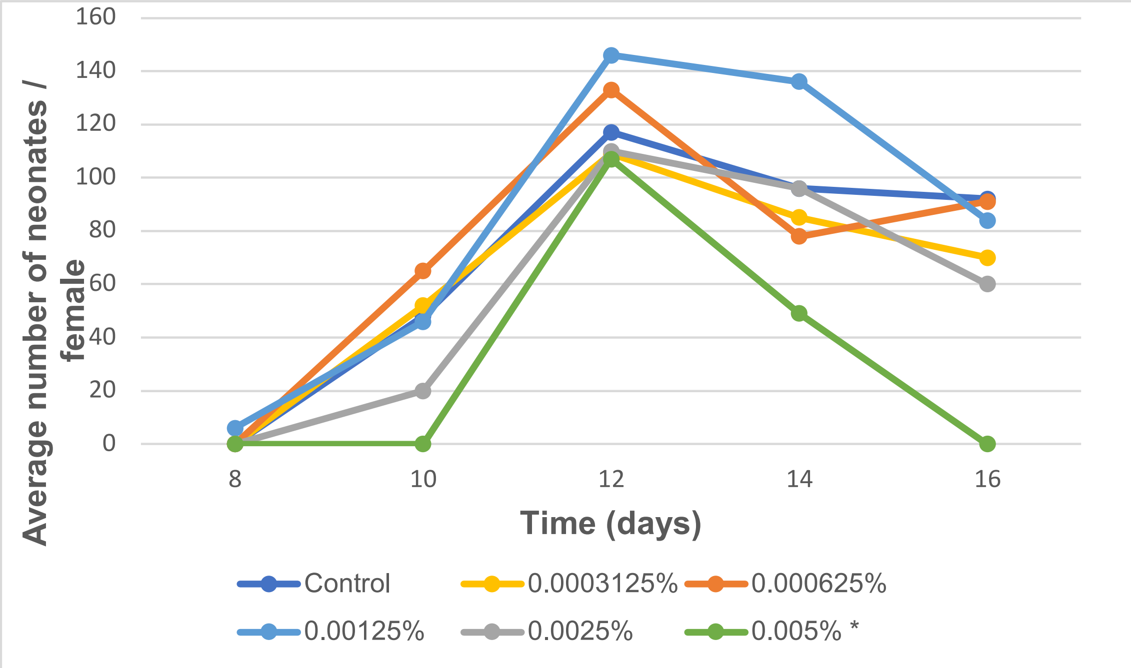

Figure 4. Time for the first brood (days) over 21 days after exposure to Liovac®. Asterisk (*) = significantly different from the control ($p < 0.05$).
Source: Elaborated by the author.

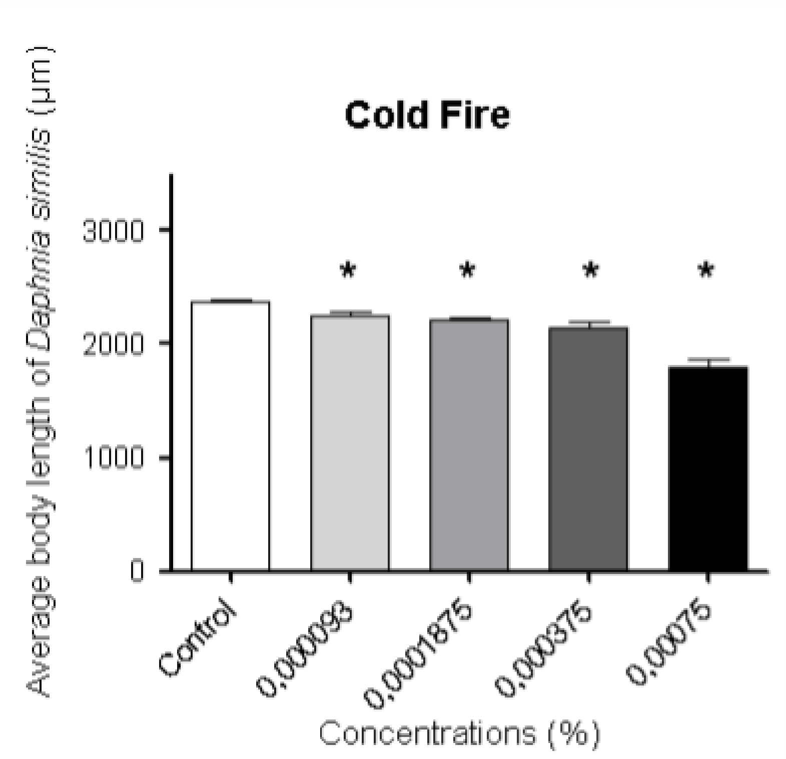

Figure 5. Average body length of *Daphnia similis* exposed to concentrations of Cold Fire®. Asterisk (*) = significantly different from the control ($p < 0.05$).
Source: Elaborated by the author.

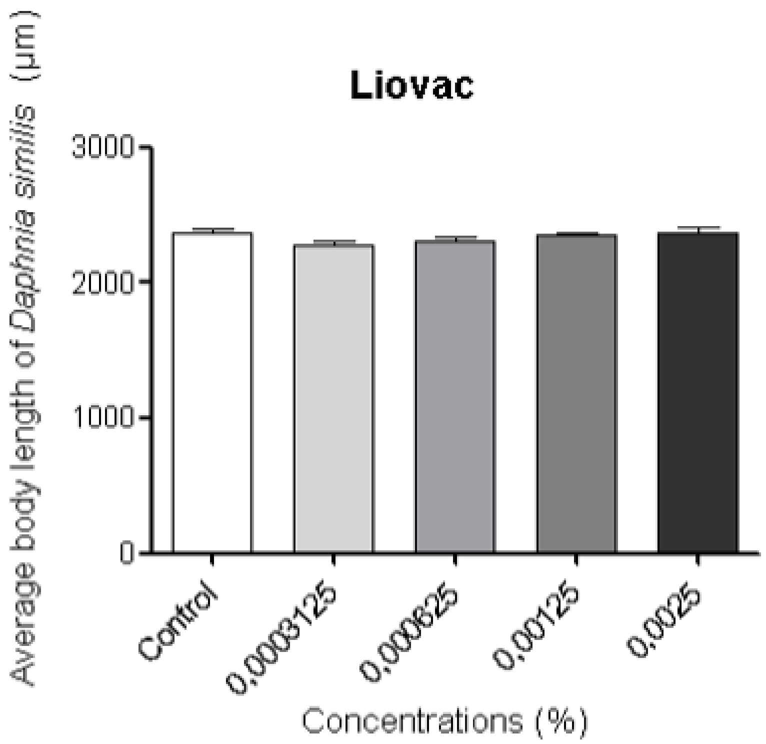

Figure 6. Average body length of *Daphnia similis* exposed to concentrations of Liovac®.
Source: Elaborated by the author.

4. DISCUSSION

The present study shows that Cold Fire® and Liovac® AFFFs caused reproductive disturbances in *D. similis*. Muyssen *et al.* (2006) and Villarroel *et al.* (2013) highlight that a way to analyze impacts at the molecular, biochemical, and physiological level is to use reproduction as a study endpoint, considering that the energy cost for an organism to reproduce, it is expressed by an increase in these costs in individuals exposed to toxic substances, which directly influences their ecological fitness, that is, the adaptive success is determined by the number of individuals produced. Our results demonstrate that long-term exposure to contaminants, even in low concentrations (0,0001875%) caused mortality of individuals, as mentioned in previous studies (LU *et al.*, 2015; DA SILVA *et al.*, 2019; DANIEL *et al.*, 2021) and effect on the reproduction of neonates as demonstrated in the present study.

Da Silva *et al.* (2019) pointed out the same commercial substances (Cold Fire® and Liovac®) causes mortality even in tests with a concentration below 1%. Daniel *et al.* (2021) verified the toxicity of Cold Fire® in tests isolated and combined with other substances and obtained negative effects in concentrations below 1%. The results obtained in this work reinforce the toxic potential of these formulations.

In addition, there was a delay in the maturation of *D. similis*, and these effects were observed only in the higher concentration (Table 2), which corroborates the results observed by Barmentlo *et al.* (2015) which proved a delay in the maturation of daphnias exposed to PFAS with increased concentrations. Jeong *et al.* (2016) performed a multigenerational test where exposure to PFOS tended to delay the time for the first brood depending on the concentration, which was observed in the five generations and supported the idea that oxidative stress is a potential mode of action for perfluorinated substances. Lu *et al.* (2015) and Yang *et al.* (2019) also found an increasing in time for first reproduction of the same species of *daphnia* exposed to PFOS, PFNAS and mixture of compounds (PFOS / PFA).

In this study, the growth of daphnids was evaluated (0,000093%; 0,0001875%; 0,000375%; 0,00075%) in all Cold Fire® exposures. The 0.0015% concentration caused mortality of all organisms until the end of the chronic test. Yi *et al.* (2010) pointed out that contaminants that can cause changes in the nervous system, such as PFOS, can interfere in the rate of normal food intake by *Daphnia*. Kim *et al.* (2010) and Rinke and Petzoldt (2003) observed that the concentration of food eaten is the principal determinant of the maximum body length of these organisms. Lu *et al.* (2015) reported a significant decrease ($p < 0.05$) in the body length of a species of *Daphnia*, due to the exposure of similar perfluorinated substances to lower concentration, which strengthens the data obtained in this study. Results observed by Yang *et al.* (2019) showed a significant decrease ($p < 0.05$) in *Daphnias* exposed to similar concentration employed in the present study.

Some protocols such as UL162 (1994) and NBR 15511 (2008), pointed out that FSA as a synthetic, non-toxic, and biodegradable product in concentrations 1%, 3% and 6%, and therefore carry an “Environmental friendly” seal (friendly to the environment), which contradicts several studies, including this one, in which negative effects were observed on organisms exposed to low concentrations, such as 0.000093%. Recent studies have mentioned the use of these compounds to fight port fires, resulted in about 12 thousand deaths of fish and invertebrates (BORGES, 2015) due to exposure of these contaminants, which was considered the greatest disaster of its kind ever recorded in Brazil until the present moment (ROTUNDO *et al.*, 2020). Da Silva *et al.* (2019) and Daniel *et al.* (2021) reported the toxic potential of perfluorinated substances on an individual basis, which are the same found in this Brazilian environmental matrix disaster, which is being reinforced by this study. However, Lu *et* *al.* (2015) and Liang *et al.* (2017) described a potential synergism of toxicity when there is a mixture of perfluorinated compounds, which suggests that the severity of the event is on a larger scale than mentioned in the literature and cannot measure the extent for local biological contamination.

A search for substitutes to long-chain perfluoroalkyl carboxylic acids (PFCAs) and their potential precursors is mentioned in the literature by short-chain homologues, composed of 6 carbons or less in their chemical composition since the early 2000s (WANG *et al.*, 2013), with the purpose of commercializing a less bioaccumulative compound than its predecessors, with the same effectiveness (GOMIS *et al.*, 2018). However, the available data on the environmental fate and ecotoxicology of these alternatives remain limited, which makes it difficult to assess and manage these chemicals (WANG *et al.*, 2013, XIAO, 2017).

5. CONCLUSION

The present study investigated toxic effects of two commercial formulations of FSAs to *D. similis* in chronic effects, such as delayed maturation of organisms and decreased reproduction, were observed, as well as reduced body length in individuals exposed to lower concentrations than those recommended by manufacturers. Cold Fire® was more toxic than Liovac®. It is necessary to consider the effects caused by PFAS before use in class B firefighting in the environment.

6. TRABALHO SUBMETIDO OU APROVADO

Dear Mr. Cara,

Thank you for submitting your manuscript,
"Could aqueous film-forming foams (AFFFs) and encapsulator agents (EAs) interferes on the reproduction and growth of Daphnia similis?", to Water, Air, & Soil Pollution

The submission id is: WATE-D-21-00660
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During the review process, you can keep track of the status of your manuscript.

Your username is: alisoncara
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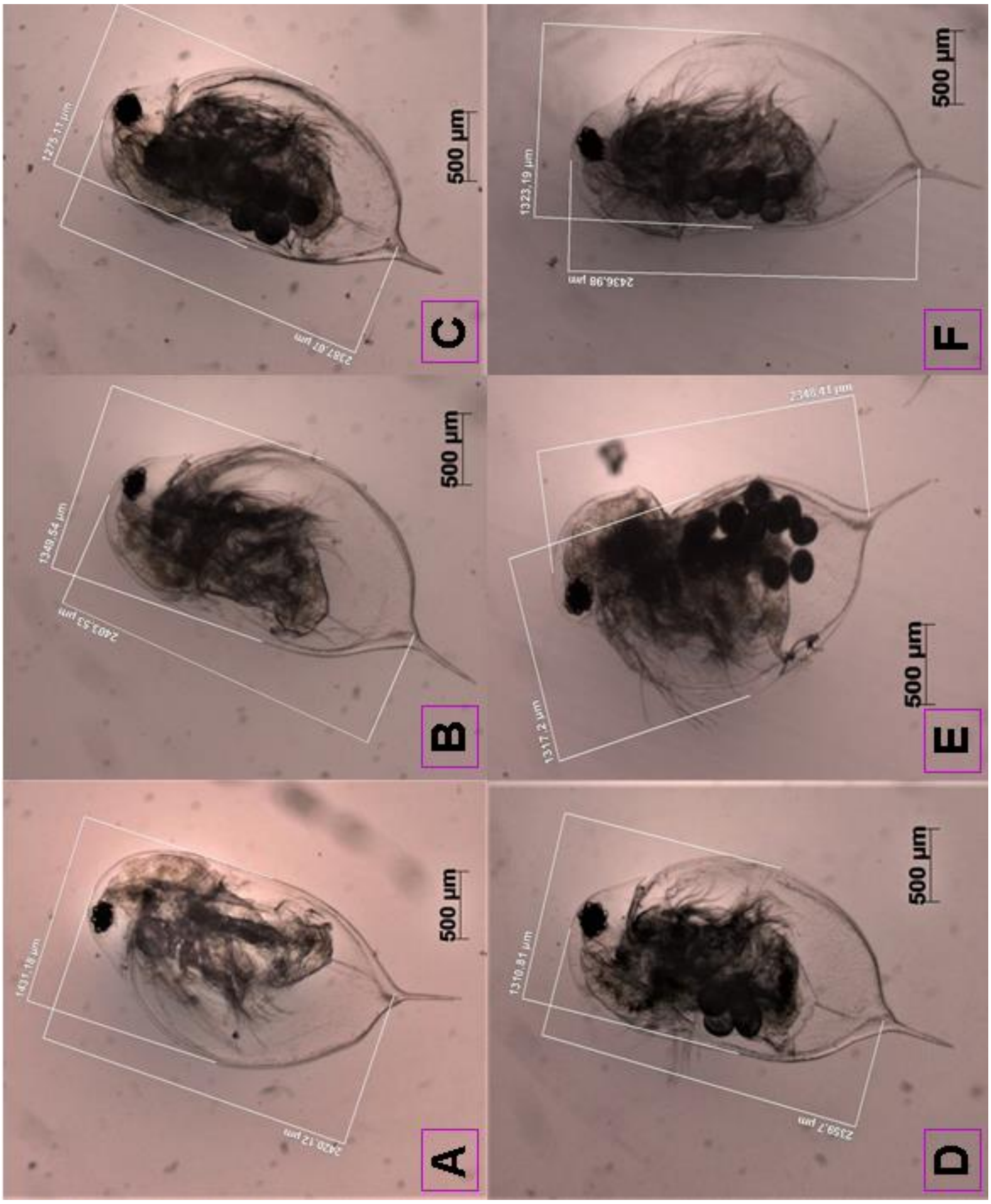
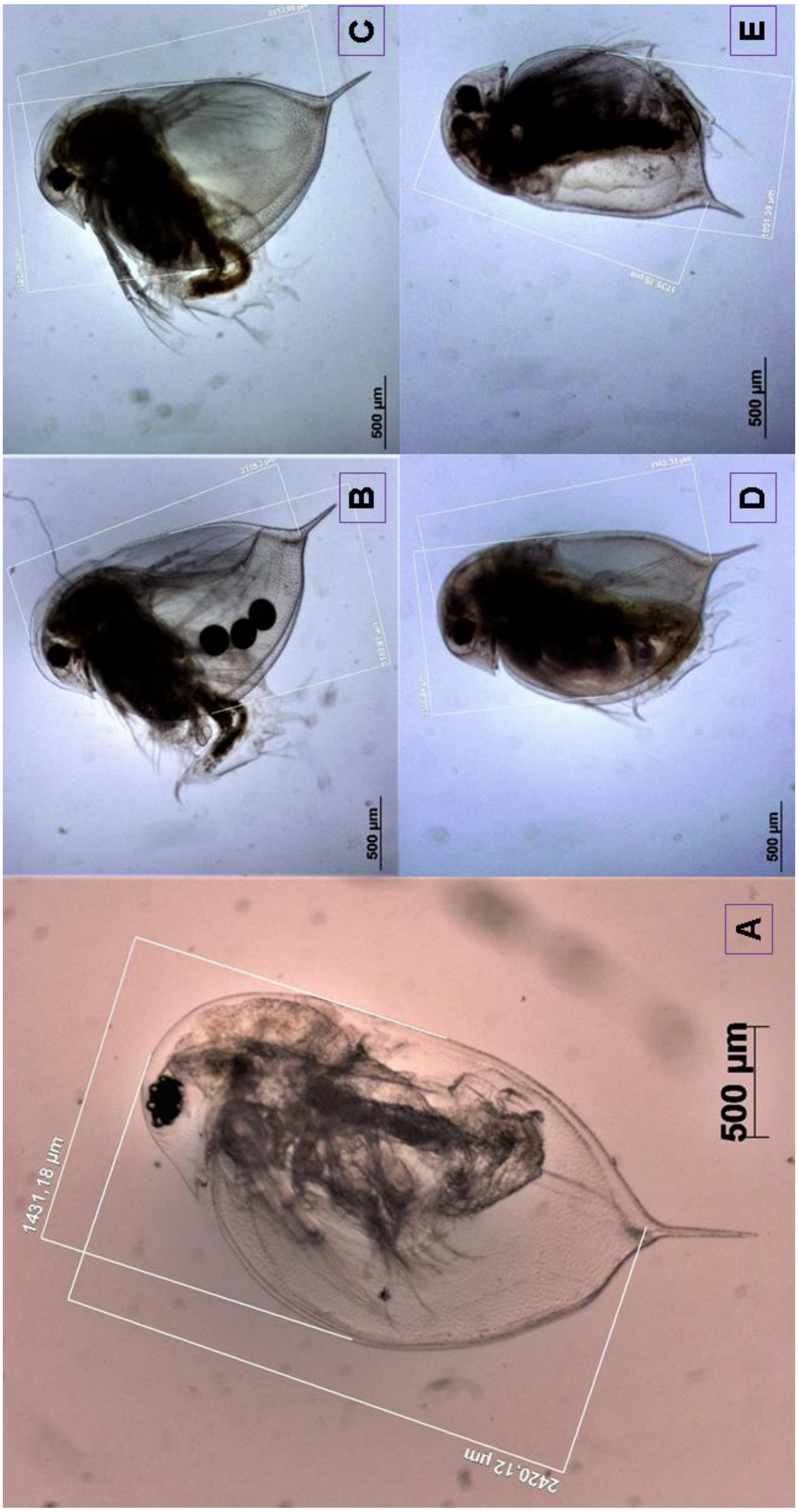
ANEXO A - Morfometria de *Daphnia similis* expostas a concentrações de Liovac® ao final de 21 dias.



(A) *Daphnia similis* control; (B) exposed to 0,0003125%; (C) exposed to 0,000625%; (D) exposed to 0,00125%; (E) exposed to 0,0025%; (F) exposed to 0,005%.

(B) Source: Elaborated by the author.

ANEXO B - Morfometria de *Daphnia similis* expostas a concentrações de Cold Fire® ao final de 21 dias.



(A) *Daphnia similis* control; (B) exposed to 0,000093%; (C) exposed to 0,0001875%; (D) exposed to 0,000375%; (E) exposed to 0,00075%.
(B) Source: Elaborated by the author.