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Assessing Caffeine and Linear Alkylbenzene Sulfonate Effects on Molting and Reproduction of *Daphnia magna* by Quantitative and Qualitative Approaches

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Abstract Caffeine (CAF) and linear alkylbenzene sulfonate (LAS) are human activity indicators, classified as contaminants of emerging concern (CECs). The long-term effects of these CECs on keystone species are still scarce in the literature. In this study, the molting and reproduction of *Daphnia magna* were evaluated over chronicle experiments by quantitative and qualitative approaches. The reported environmental concentrations (scenario 1) of CAF (0.005, 0.03, and 0.127 mg L^{-1}) and LAS (0.4, 1.0, and 2.5 mg L^{-1}) did not show statistical differences in molting process. Inhibition for molting index ($\%I_M$) was observed in expected effect concentration exposures (scenario 2) to CAF (20, 40, and 60 mg L^{-1}) and LAS (4.1, 4.5, and 4.9 mg L^{-1}). A decrease in the number of offspring (17 to 30%) and anticipation of the release time of the first four broods were observed in exposures to CAF. Moreover, LAS increased the offspring number produced per *D. magna* in the 1st (33 to 40%) and 2nd (22 to 52%) broods, in addition to a reduction of the time between 2nd and 3rd broods. Evidence of offspring induction in offspring index ($\%I_O$) was observed in exposures to LAS in scenario 1 and inhibition was recorded for scenario 2 (all LAS concentration and 60 mg L^{-1} of CAF). In scenario 2, for CAF and LAS, caused an inhibition on $\%I_O$ and a significant decrease in the total offspring produced, especially on the 2nd brood (from 26 to 48%). These findings suggest that the *D. magna* life cycle may be impacted by a wide range of doses of environmentally relevant pollutants, whereas different approaches support interpreting the level of damage to daphnids' reproduction and development.

Keywords Molting effect index · Offspring effect index · Neonates · Long-term exposures · Freshwater pollution · Ecotoxicology

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1 Introduction

The contamination of aquatic environments by human activities concerns different study areas (e.g., social and environmental sciences, ecology, and economy), which demands an environmental health assessment to evaluate the effects on the aquatic biota (Corbi et al., 2008; Reddy & Behera, 2006; Schaider et al., 2019). Contaminants of emerging concern (CECs) are substances, synthetic or natural, found in personal hygiene products, medicines, and foodstuffs, among others, that reach water bodies through the incorrect disposal or domestic and industrial wastewater sewage (Hu et al., 2018; Luo et al., 2014; Sauvé & Desrosiers, 2014), and in this sense, both caffeine (CAF) and linear alkylbenzene sulfonate (LAS) are described as CEC (Brack et al., 2012; NORMAN, 2016).

CAF is the most widely consumed stimulant drug worldwide, commonly used as an active ingredient in commercial drugs to enhance mental and physical performance (Kolpin & Meyer, 2002; Snyder et al., 2007). This substance is also present in coffee, tobacco, spices, teas, sodas, and many other foods (Temple et al., 2017). However, CAF is not fully metabolized in the human body, and it can be eliminated in wastewater and reach the environment (Wang et al., 2011). Benotti and Brownawell (2009) observed that CAF half-life ranged from 3.5 to more than 100 days depending on the water's physical and chemical characteristics. The continuous intake of CAF from anthropogenic sources raises its environmental concern (Daneshvar et al., 2012; Machado et al., 2016) that can be worsened by the increase of caffeine consumption in areas such as Europe (Quadra et al., 2020). LAS is a primary surfactant used in laundry detergent formulations worldwide, tending to be absorbed into suspended solids and accumulated in sediments (Zhu et al., 1998). Due to the amphiphilic nature of its carbon chain and its sulfonate group, this substance can be solubilized and easily transported by water (Hampel et al., 2012; Prats et al., 1993). Environmental contamination by LAS has been reported in many environmental compartments in concentrations from part per trillion to part per billion (Eichhorn et al., 2002; Sanderson et al., 2006) and the increase in the projected global market of surfactant demonstrates that the fate, distribution, and persistence of surfactants in the environment is an urgent matter (Badmus et al., 2021).

Both CAF and LAS are water quality indicators due to their constant presence in domestic wastewater, seawater, streams, groundwater, and the direct association with human activity (Brack et al., 2012; Buerge et al., 2006; Gonçalves et al., 2017; Moore et al., 2008; NORMAN, 2016). CAF was detected from 3.4 to 6.6 μg L^{-1} in effluents from wastewater treatment plants (WWTPs), reaching up to 127 μg L^{-1} in water bodies (Montagner & Jardim, 2011; Sui et al., 2010), while LAS occurred in WWTPs ranging from 0.4 to 2.5 mg L^{-1}, and in streams with concentrations up to 1 mg L^{-1} (Eichhorn et al., 2002; Fox et al., 2000; Holt et al., 1998; Knepper et al., 2003). In this scenario, ecotoxicological assessment contributes to the environmental water quality monitoring, aiming to preserve the environment against adverse biological effects from multiple chemical contaminations (Altenburger et al., 2019; Hoogenboom et al., 1999).

The microcrustacean *Daphnia magna* Straus 1820 (Crustacea: Cladocera) has been used as a bioindicator since 1940 and stabilized as a standard organism-test in ecotoxicity assays in the 1970s (Anderson, 1944; Anderson & Jenkins, 1942). Offspring, juveniles, and adults compose the organism's life cycle, and during the development, the organisms exchange their ecdysis (molting process), which allows them to grow and release their neonates. The molting process is controlled by the ecdysteroid hormones and can be influenced by CEC, including hormones and endocrine substances (Giraudo et al., 2017; Martin-Creuzburg et al., 2007; Sumiya et al., 2014).

The *Daphnia magna* reproduction test guideline is described by the OECD (2012) and considered as endpoints: the number of living offspring produced by each parent animal; the possible calculation of the concentration associated with x% of effect (EC_x); the lower observed effect concentration (LOEC); and the no observed effect concentration (NOEC). Although these endpoints bring reference values for specific characteristics of the organism's life cycle, there is a lack of qualitative indicators showing other chemical effects. During the *Daphnia* reproduction test, it is possible to record complementary parameters to the offspring number, including the time of the first molting, the frequency and number of molting throughout each organism's life, and the association between molting and reproduction.

Although some CECs present low acute toxicity, these contaminants may cause significant reproductive effects even at low exposure levels (OECD, 2008). Nonetheless, these chemicals can have specific modes of action that may affect *D. magna*. Thus, analyzing qualitative endpoints can complement the investigation of toxic effects and evidence the *D. magna* life history strategy in response to environmental stressors. In this sense, we assessed the impact of CAF and LAS on the reproduction and molting processes of *D. magna*. Two scenarios were evaluated based on the (1) reported environmental concentrations and (2) expected effect concentrations. From the demand for further research on emerging pollutants, distinct approaches are fundamental to bring additional information regarding the organism's responses to these pollutants. To evaluate this gap, we included qualitative indexes to the conventional quantitative endpoints.

2 Materials and Methods

2.1 *D. magna* Cultivation

According to the OECD Guidelines (OECD, 2012), daphnids used in the test were cultivated at the Aquatic Ecology Laboratory, at São Carlos School of Engineering, University of São Paulo, Brazil. The organism's health status was assessed every 2 months with sensitivity bioassays using copper sulfate pentahydrate ($CuSO_4.5H_2O$, Synth®) as the reference substance (ABNT, 2016).

2.2 Exposure Design

The selected environmental pollutants were CAF ($C_8H_{10}N_4O_2$) (Sigma-Aldrich®, > 99% of purity) and LAS ($C_{12}H_{25}C_6H_4SO_3Na$) (Sigma-Aldrich®, 80.7% of purity). The stock solutions (nominal concentrations) were prepared in distilled water (LAS 1.0 g L^{-1} and CAF 100 mg L^{-1}). Two scenarios were proposed based on the literature: (1) reported environmental concentrations; (2) expected effect concentrations (Table 1).

The *D. magna* reproduction test was adapted from the OECD Guidelines (2012) and the test was carried out at a controlled temperature (20 ± 2 °C), and under 16 h light:8 h darkness. Reconstituted water was prepared as a mineral solution of $CaCl_2.2H_2O$ (73.5 g L^{-1}), KCl (5.8 g L^{-1}), $NaHCO_3$ (64.8 g L^{-1}), and $MgSO_4.7H_2O$ (123.3 g L^{-1}) dissolved in distilled water under constant aeration (ABNT, 2016). The hardness (175–225 mg $CaCO_3$ L^{-1}) and the pH (from 7.6 to 8.0) were controlled. *Daphnia magna* was fed with yeast suspension (0.125 mg L^{-1}), fish food (2.5 mg L^{-1}), and microalgae (*Raphidocelis subcapitata*) grown in L.C. Oligo medium (3 × 10^5 cells mL^{-1}) (ABNT, 2011).

A test control with only reconstituted water was used to evaluate the life cycle of *D. magna* without contamination. This test compared the daphnids' ability to respond to environmental changes in the molting cycle and reproduction ratio. The reproduction test had 10 replicates that consisted of one neonate (< 24 h old) in a 100 mL beaker for each treatment (CAF and LAS concentrations, and control). The test was operated in semi-static conditions over 21 days, with the test solution's renew and feeding three times a week in all replicates. The pH and conductivity of all treatments were measured weekly.

The parental immobility, occurrence of offspring, and molting were recorded daily. Throughout the assays, the offspring, molts, and immobile organisms were removed. Regarding the molting process from a quantitative approach, we analyzed CAF and LAS effects on the number of molts produced per parental

Table 1 Reported environmental concentrations (scenario 1) and expected effect concentrations (scenario 2) selected for *Daphnia magna* reproduction tests

Treatment	CAF (mg L^{-1})[a]	LAS (mg L^{-1})[b]
Laboratory control	0.0	0.0
Scenario 1	0.005	0.4
	0.03	1.0
Reported environmental concentrations	0.127	2.5
Scenario 2	20	4.1
	40	4.5
Expected effect concentrations	60	4.9

[a]Busse and Nagoda (2015); Cantwell et al. (2016); Chen et al. (2002); Edwards et al. (2015); Gonçalves et al. (2017); INCTAA (2014); Jagoda et al. (2015); Kolpin and Meyer (2002); Machado et al. (2016); Moore et al. (2008); Silva et al. (2014); Sposito et al. (2018); Sui et al. (2010); You et al. (2015)

[b]Atici (2021); Fox et al. (2000); Holt et al. (1998); Knepper et al. (2003); Sanderson et al. (2006); van de Plassche et al. (1999); Verge et al. (2001)

D. magna and the number of molts produced until the primiparity stage (day of first brood release) and intermolt time. As outputs of the reproductive process, we analyzed the effects on offspring produced per parental *D. magna*, offspring produced per brooding (counting the first three broods in all CAF and LAS treatments and control), broods release time (the first day of exposure as starting time, with comparisons until the 4th brood for all treatments and control), and time between broods (considering the four first brood in all CAF and LAS treatments and control).

2.3 Statistical Analysis

The Shapiro-Wilk normality test (significance level $p = 0.05$) was applied to identify the type of data distribution. For data that showed normal distribution ($p \geq 0.05$), the one-way ANOVA test was performed to investigate differences between CAF or LAS treatments and control. The Fisher's test was applied to identify the specific differences pointed out by the previous test. Comparisons of independent samples by groups, such as scenarios 1 and 2 endpoints, were analyzed using the *T*-test. Regarding non-normal data analysis ($p < 0.05$), significant differences between CAF or LAS treatments and control were based on the Kruskal-Wallis test, followed by the two-tailed test. Moreover, comparisons of independent samples by groups were processed by the Mann-Whitney test. The statistical treatment of data was executed using Statistica® software version 7 and sensitivity bioassays by R® software version 3.3.5, packages MASS and DCR (R Core Team, 2018). Significant differences were considered when $p \leq 0.05$, assuming a 95% confidence interval. The significant *p*-values are presented. The responses of the selected endpoints were plotted on graphs using OriginPro® software version 8.0.

2.4 Qualitative Indexes

We proposed additional endpoints on already established ecotoxicological assays to further comprehend the ability of *D. magna* to respond to a stressful environment. To evaluate these potential effects in the daphnids' life cycle, we suggested calculating two indexes: molting effect index for the molting process (M) as $\%I_M$ and offspring effect index for offspring production (O) as $\%I_O$ index. The molts and the offspring were assessed daily for 21 days to calculate these parameters efficiently and minimize errors in the indexes. Furthermore, the cumulative number of molts corresponded to the measured value for each replicate ($[M_1, M_2, \ldots, M_n]$, where n = number of replicates) and the cumulative offspring produced for each replicate ($[O_1, O_2, \ldots, O_n]$, where n = number of replicates).

The last molt produced by a dead parental organism was used to calculate the offspring's average or molt. If the parent died producing the sum of 3 molts and 15 offspring, this value was used to calculate the indexes on the 21st day, adding the mortality/immobility effect variation from the indexes' replicates. The $\%I_M$ (Eq. 1) and $\%I_O$ (Eq. 2) indexes compare the effects of the environmental pollutants (CAF and LAS) and the test control calculated as an adaption of Sierra-Alvarez and Lettinga (1991) as follows:

$$\%I_M = \left[\left(M_{EP} - M_{TC}\right)/M_{TC}\right] \times 100 \qquad (1)$$

where:

M_{EP} average number of molts measured over 21 days for each environmental pollutant concentration, average $[M_1, M_2, \ldots, M_n]$, $n = 10$ replicates;

M_{TC} average number of molts measured over 21 days for each replicate of test control.

$$\%I_O = \left[\left(O_{EP} - O_{TC}\right)\right] \times 100 \qquad (2)$$

where:

O_{EP} average of total offspring number measured over 21 days for each environmental pollutant concentration, average $[O_1, O_2, \ldots, O_n]$, n = 10 replicates;

O_{TC} average of total offspring number measured over the 21 days of the assay for test control.

The effects of environmental pollutants on the molting process ($\%I_M$) and production of offspring ($\%I_O$) were summarized in five categories: "Induction," "Evidence of induction," "No effect," "Evidence of inhibition," and "Inhibition" (Table 2). The category "No effect" corresponds to 20% of the confidence range considering inadvertent parental mortality or accidental death (OECD, 2012) and standard

Table 2 Classification of the effect of the molting process (%I_M) and offspring production (%I_O)

%I_M or %I_O	Category
From +50 to +100	Induction
From +20 to +50	Evidence of induction
From -20 to +20	No effect
From -50 to -20	Evidence of inhibition
From -100 to -50	Inhibition

deviation of offspring production between similar conditions (10 individual L^{-1}, temperature between 20 and 25 °C) (Olkova et al., 2017).

3 Results

In general, throughout the *D. magna* reproduction test, the average pH ranged from 7.6 to 8.0. The average conductivity was 426.5, 435.2, and 509.0 μS cm^{-1} for the test control, CAF, and LAS treatments, respectively. The test control followed the OECD recommendations (OECD, 2012), with a minimum of 60 offspring production, immobility below 20%, and coefficient variation below 25% for offspring production. The *D. magna*'s health status was evaluated in sensitivity bioassays ($CuSO_4.5H_2O$), presenting EC_{50} of 0.0445 and 0.0453 mg L^{-1}, comparable to values found in the literature (0.0546 mg L^{-1}) that assure reproducible data (Struewing et al., 2015).

Parental *Daphnia* from CAF treatments scenario 1 and 20 and 40 mg L^{-1} in scenario 2 for CAF presented 20% of immobility or less, resulting in no negative effect over *D. magna* mobility. In contrast, immobility of 90% was recorded for 60 mg L^{-1} of CAF. Exposures to LAS showed that the immobility of parental *Daphnia* was dependent on the dose increase. LAS treatments from scenario 2 induced high immobility (90% at the 4.9 mg LAS L^{-1}, and 80% at 4.1 mg LAS L^{-1}). On the other hand, no immobility was recorded for LAS treatments from scenario 1 (from 0.4 to 1.0 mg LAS L^{-1}).

3.1 Effects on Molting

3.1.1 *Number of Molts Produced per Parental D. magna*

The total number of molts produced over 21 days ranged from 75 in control, and among CAF treatments, 70 (0.005 mg L^{-1} of CAF) to 78 (0.127 mg L^{-1} of CAF) for scenario 1, and from 49 (60 mg L^{-1} of CAF) to 75 (20 mg L^{-1} of CAF). In LAS exposures, the total number of molts varied between 69 (1 mg L^{-1} of LAS) and 75 (0.4 mg L^{-1} of CAF), in scenario 1. For scenario 2, it ranged from 16 (4.9 mg L^{-1} of LAS) to 41 (4.1 mg L^{-1} of LAS). Fig. 1 shows the number of molts produced per parental *Daphnia* in 21 days according to exposure scenarios.

No statistical differences were identified between CAF treatments (scenario 1) and control, and between LAS treatments (scenario 1) and control in the number of molts produced by parental *Daphnia* throughout the exposures ($p > 0.05$, Kruskal-Wallis test). However, for expected effect concentrations (scenario 2), significant differences were observed comparing CAF treatments and control ($p = 0.005$) and LAS treatments and control ($p = 0.0004$), according to the Kruskal-Wallis test. The two-tailed test revealed that the number of molts in 60 mg L^{-1} of CAF (5 ± 2 molts) and 4.9 mg L^{-1} of LAS (2 ± 2 molts) were statistically different from the control (8 ± 1 molts) (CAF: $p = 0.008$ and LAS: $p = 0.0002$, respectively). Comparing the number of molts produced per parental *Daphnia* between scenarios, the Mann-Whitney test pointed out no differences between CAF treatments scenarios 1 and 2 ($p > 0.05$), in contrast to that

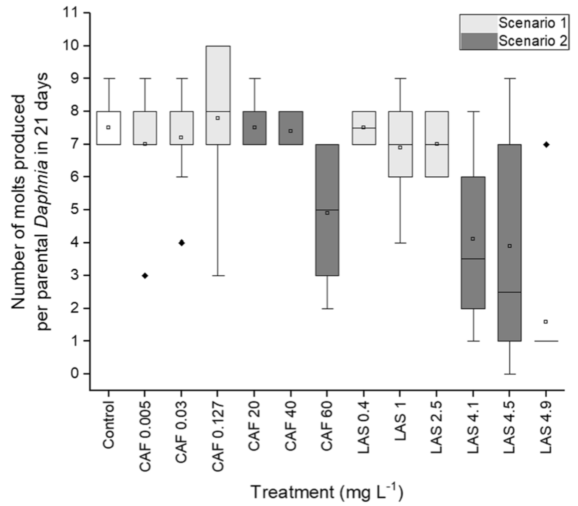

Fig. 1 Number of molts produced per parental *Daphnia magna* over 21 days

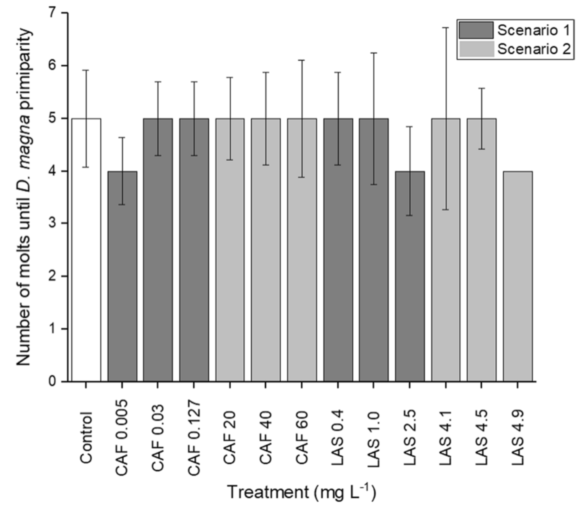

Fig. 2 Number of molts until *D. magna* primiparity

observed between LAS treatments scenarios 1 and 2, which showed a significant difference ($p = 0.000003$).

3.1.2 Molting Process Until Primiparity

Except for the 0.005 mg L^{-1} of CAF, 2.5 mg L^{-1} of LAS, and 4.9 mg L^{-1} of LAS treatments, parental *Daphnia* reached primiparity by producing an average of 5 molting (Fig. 2). There were no significant differences between CAF or LAS treatments and control, considering scenarios 1 and 2 ($p > 0.05$, Kruskal-Wallis test). Besides, comparing the number of molts until the first reproduction between CAF treatments from scenarios 1 and 2, and LAS treatments from scenarios 1 and 2, no statistical differences were identified ($p > 0.05$, Mann-Whitney test).

3.1.3 Intermolt Time

In general, the average intermolt time varied from 2 to 3 days in control, CAF, and LAS treatments. Fig. 3 shows the intermolt time of parental *Daphnia* in the experiments. According to the Kruskal-Wallis test, statistical differences were pointed out between CAF treatments (scenario 2) and control ($p = 0.03$), and the 60 mg L^{-1} of CAF treatment showed a significant difference compared to the control ($p = 0.02$, two-tailed test). Statistical similarities were observed between CAF treatments (scenario 1) and control, and between LAS treatments (scenarios 1 and 2) and control ($p > 0.05$, Kruskal-Wallis test). Comparing the intermolt time between CAF treatments from scenarios 1 and 2, and LAS treatments from scenarios 1 and 2, no significant differences were found ($p > 0.05$, Mann-Whitney test).

3.1.4 Molting Effect Index

From the %I_M data, only the 60 mg L^{-1} of CAF treatment presented a negative response to environmental

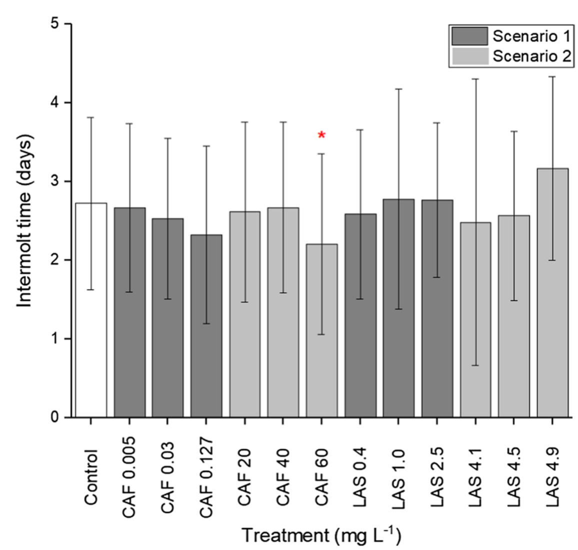

Fig. 3 *D. magna* intermolt time

changes in the molting cycle ratio, classified as "evidence of inhibition" (Table 3). The 0.005 to 40 mg L^{-1} of CAF treatments did not affect the molting process ("no effect"). Besides, the $\%I_M$ of LAS showed concentration-dependent effects on the *D. magna* life molting cycle. There was no evidence of induction or inhibition for 0.4 to 2.5 mg LAS L^{-1} treatments, classified as "no effect." Nevertheless, higher concentrations of LAS produced inhibition response, specifically for 4.1 and 4.5 mg LAS L^{-1}, which resulted in "evidence of inhibition" classification, and for 4.9 mg L^{-1}, where an "inhibition" effect was determined.

3.2 Effects on Reproduction

3.2.1 Offspring Produced per Parental D. magna

Over 21 days, parental daphnids from the control test produced a total of 709 offspring. For CAF reported environmental concentrations (scenario 1), the total offspring varied between 603 (0.0005 mg L^{-1}) and 760 (0.127 mg L^{-1}). In the CAF scenario 2 (expected effect concentrations), the range of total offspring produced was between 199 (60 mg L^{-1}) and 732 (40 mg L^{-1}). In LAS treatments, the total offspring produced ranged from 865 (1 mg L^{-1}) to 963 (2.5 mg L^{-1}) for scenario 1, and from 67 (4.9 mg L^{-1}) to 191 (4.5 mg L^{-1}) for scenario 2. Fig. 4 shows the number of offspring produced per parental *Daphnia* in 21 days according to exposure scenarios.

No statistical differences were observed in the number of offspring produced by parental *Daphnia* between CAF treatments and control for scenario 1

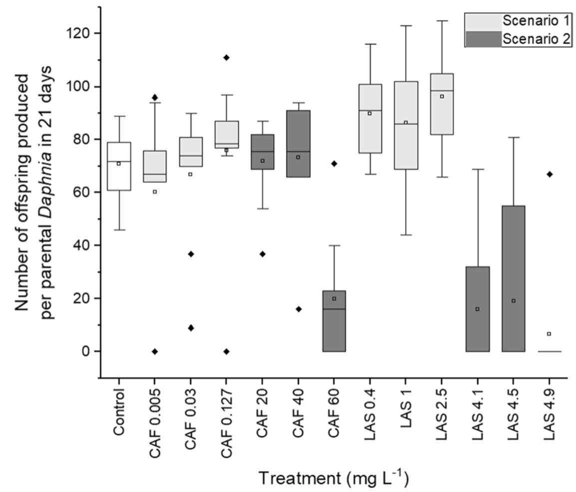

Fig. 4 Number of offspring produced per parental *D. magna* over 21 days

($p > 0.05$, Kruskal-Wallis test). For scenario 2, differences between CAF treatment and control were pointed out ($p = 0.0005$, Kruskal-Wallis test). According to the two-tailed test, the number of offspring produced by parental *Daphnia* in the 60 mgL^{-1} of CAF treatment showed a statistical difference with the control ($p = 0.01$). Moreover, no statistical difference was observed in the number of offspring produced between scenarios 1 and 2 in CAF exposures ($p > 0.05$, Mann-Whitney test).

Statistical differences were observed between LAS treatments and control for scenario 1 ($p = 0.02$, Kruskal-Wallis test), and this difference occurred between 2.5 mg L^{-1} and control ($p = 0.01$, two-tailed

Table 3 Molting effect index ($\%I_M$) calculated for CAF and LAS exposures in scenarios 1 and 2

CAF - $\%I_M$ (molting effect index)			LAS - $\%I_M$ (molting effect index)		
Scenario 1	CAF 0.005	-13.8%	Scenario 1	LAS 0.4	-10.1%
	CAF 0.03	-10.1%		LAS 1.0	-15.1%
	CAF 0.127	-0.3%		LAS 2.5	-15.1%
Scenario 2	CAF 20	-7.7%	Scenario 2	LAS 4.1	-44.6%
	CAF 40	-11.4%		LAS 4.5	-43.4%
	CAF 60	-39.7%		LAS 4.9	-79.1%

test). For LAS scenario 2, statistical differences were identified between LAS treatments and control (p = 0.0001, Kruskal-Wallis test). The 4.1, 4.5, and 4.9 mg L^{-1} treatments showed statistical difference with control (p = 0.008, p = 0.01, and p = 0.001, respectively), according to the two-tailed test. The number of offspring produced in scenario 1 was statistically different from scenario 2, in LAS exposures (p < 0.05, Mann-Whitney test).

3.2.2 Offspring Produced per Brooding

Regarding the number of broods, the first four broods were registered in the control test, 40, 60 mg L^{-1} of CAF, and 1, 2.5, 4.1, 4.5, and 4.9 mg L^{-1} of LAS. In the treatments 0.005, 0.03, 0.127, and 20 mg L^{-1} of CAF and 0.4 mg L^{-1} of LAS, parent daphnids reached 5 broods. Fig. 5 shows the number of offspring per brood in CAF (a) and LAS (b) exposures. In the 1st brood, there were no significant differences in the number of offspring between CAF treatments (scenario 1) and the control, and CAF treatments (scenario 2) and control (p > 0.5, according to the one-way ANOVA test). Statistical differences between LAS treatments (scenario 1) and control were pointed out (p = 0.03, one-way ANOVA test). According to Fisher's test, there are differences in the number of offspring produced per parental *Daphnia* between 1, 2.5 mg L^{-1} of LAS, and control (p = 0.02 and p = 0.01, respectively), in which there was an increase in the average number of offspring from the 1st brood (33 to 40%) compared to the control. In contrast, there were no differences between LAS treatments (scenario 2) and control (p > 0.05, one-way ANOVA test).

In the 2nd brood, statistical differences were observed between CAF treatments and control for both scenarios (p = 0.02, one-way ANOVA test). Fisher's test pointed out differences between 0.005, 0.03, and 0.127 mg L^{-1} of CAF and control (p = 0.003; p = 0.04; and p = 0.04, respectively) for scenario 1, showing a decrease in the average number of offspring (17 to 30%), compared to the control. Differences between 60 mg L^{-1} of CAF and control (p = 0.01) for scenario 2 were also identified on Fisher's test (39% of offspring decrease in this CAF treatment). Similarly, there are differences between LAS treatments and control for scenarios 1 and 2 (p = 0.0001 and p = 0.02, respectively, one-way ANOVA test). According to the Fisher's test, the number of offspring produced in 1 and 2.5 mg L^{-1} of LAS treatments (scenario 1), and 4.1, 4.5, and 4.9 mg L^{-1} of LAS treatments (scenario 2) showed statistical difference with control (p = 0.02, p = 0.000008, p = 0.04, p = 0.03, and p = 0.02, respectively). For these statistically significant treatments, an increase in the average number of offspring from 22 to 52% (LAS treatments, scenario 1) and a decrease of 26 to 48% (LAS treatments, scenario 2) were observed.

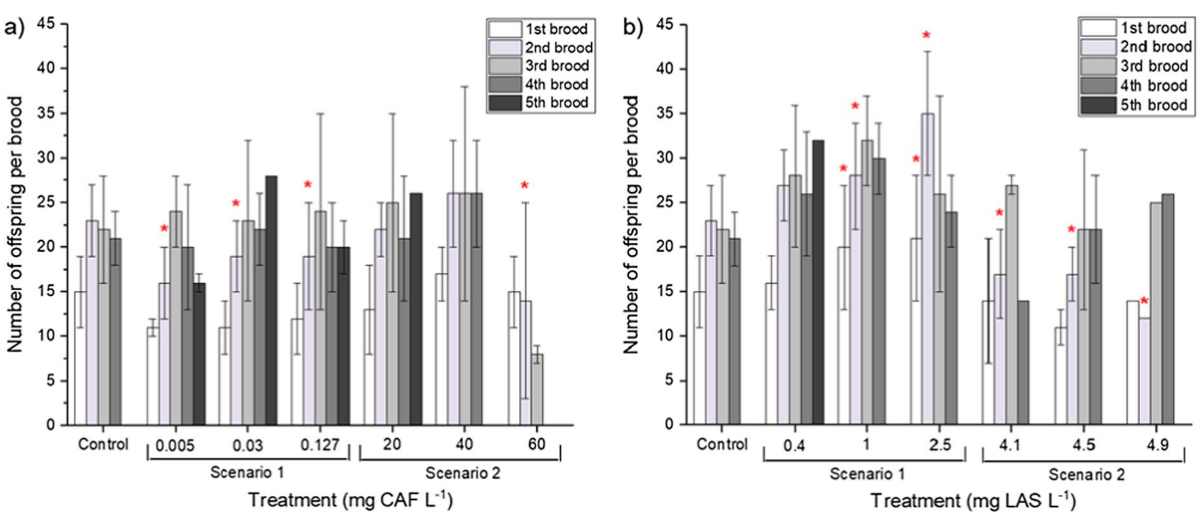

Fig. 5 Number of offspring per daphnids brood: **a** CAF treatments; **b** LAS treatments

Regarding the 3rd brood, the number of offspring, whether produced in the CAF or LAS treatments, showed no significant differences with the control for both scenarios ($p > 0.5$, one-way ANOVA test). Comparing the number of offspring produced per parental *Daphnia* between scenarios in each brood posture (*T*-test), statistical differences were identified between CAF treatments of scenarios 1 and 2, and LAS treatments of scenarios 1 and 2 for 1st brood (CAF: $p = 0.004$ and LAS: $p = 0.01$), and 2nd brood (CAF: $p = 0.006$ and LAS: $p = 0.000003$), respectively. Nevertheless, there were no differences between scenarios considering the number of offspring from the 3rd broods of CAF and LAS treatments ($p > 0.05$), according to the *T*-test.

3.2.3 Broods Release Time

Fig. 6 shows the brood release time of parental *Daphnia* in CAF and LAS exposures. Significant differences were observed between CAF treatments of scenario 1 and the control, for the 1st ($p = 0.03$), 2nd ($p = 0.01$), 3rd ($p = 0.003$), and 4th ($p = 0.03$) broods, according to the Kruskal-Wallis test. The two-tailed test pointed out that the release time for the 3rd brood in 0.005 mg L^{-1} of CAF treatment differs from the time recorded in control ($p = 0.04$), highlighting an average reduction of 2 days of the offspring's posture. We observed anticipation of the brood release (an average of 1 to 3 days, compared to the control) for 0.127 mg L^{-1} of CAF in the first four broods (1st brood, $p = 0.05$; 2nd brood, $p = 0.02$; 3rd brood, $p = 0.004$; 4th brood, $p = 0.02$), according to the two-tailed test. In CAF treatments scenario 2, and LAS treatments scenarios 1 and 2, no statistical differences were identified comparing each brood's release time and the control ($p > 0.05$, Kruskal-Wallis test). The Mann-Whitney test showed that there are significant differences in brood release time of parental *Daphnia* from CAF treatments between scenarios 1 and 2 for the 1st ($p = 0.00005$), 2nd ($p = 0.00003$), and 3rd ($p = 0.005$) broods. For the 4th brood of CAF and LAS treatments, no significant differences were identified between scenarios 1 and 2 ($p > 0.05$, Mann-Whitney test).

3.2.4 Time Between Broods

The elapsed time between 1st–2nd broods, 2nd–3rd broods, and 3rd–4th broods is presented in Fig. 7. Significant differences between treatments and control were only for LAS scenario 1 ($p = 0.009$, Kruskal-Wallis). The elapsed time between 2nd and 3rd broods in 1 mg LAS L^{-1} showed significant differences with the control (on average 1 day less than the standard registered in the control), $p = 0.02$, two-tailed test. There were no significant differences

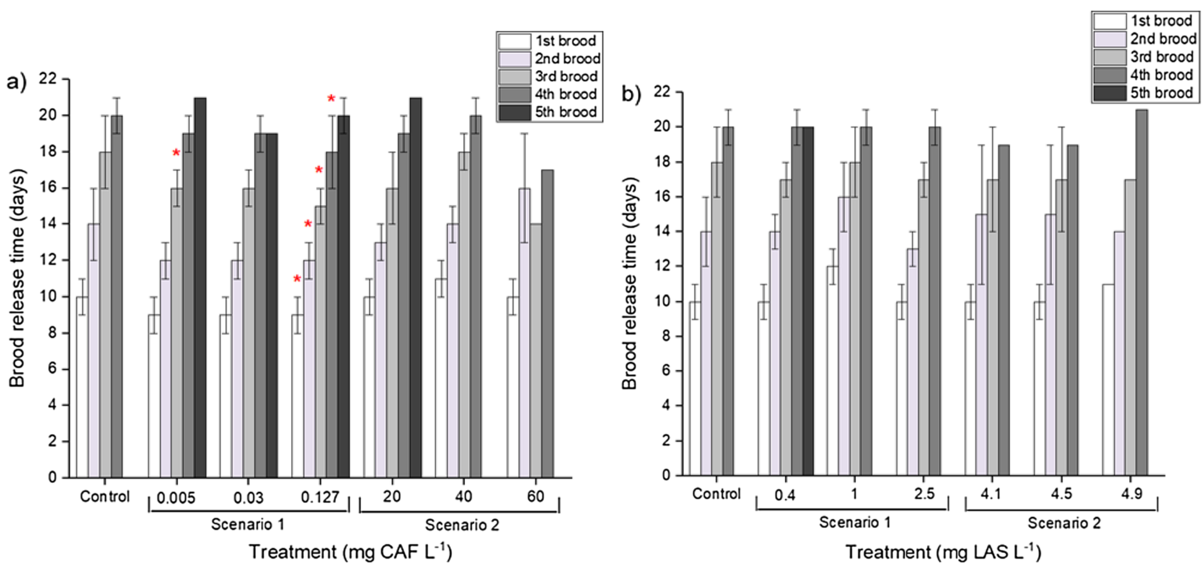

Fig. 6 Brood release time: **a** CAF treatments; **b** LAS treatments

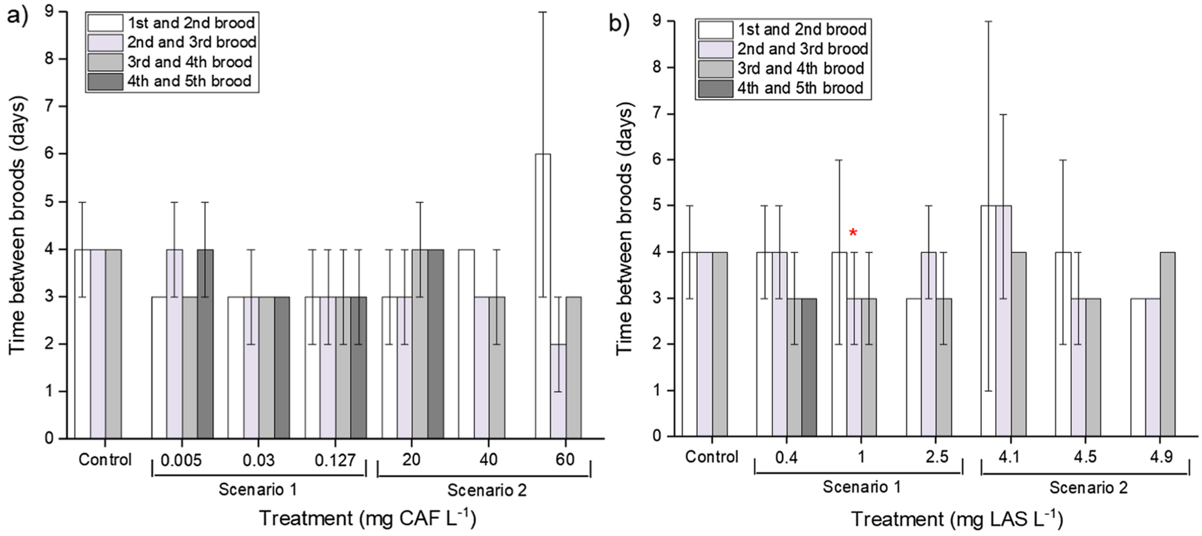

Fig. 7 Time between broods: **a** CAF treatments; **b** LAS treatments

Table 4 Offspring effect index (%I_O) calculated for CAF and LAS exposures in scenarios 1 and 2

CAF - %Io (offspring effect index)			LAS - %Io (offspring effect index)		
Scenario 1	CAF 0.005	-13.1%	Scenario 1	LAS 0.4	+29.4%
	CAF 0.03	-4.2%		LAS 1.0	+24.6%
	CAF 0.127	+9.6%		LAS 2.5	+38.8%
Scenario 2	CAF 20	+3.9%	Scenario 2	LAS 4.1	-75.8%
	CAF 40	+1.4%		LAS 4.5	-65.0%
	CAF 60	-71.3%		LAS 4.9	-90.3%

between treatments and control for CAF scenarios 1 and 2, and LAS scenario 2 (p > 0.05. Kruskal-Wallis). According to the Mann-Whitney test, comparing CAF treatments scenarios 1 and 2, only the time between 1st and 2nd broods was statistically different (p = 0.001). No significant differences compared the time considering LAS treatments scenarios 1 and 2 (p > 0.05, Mann-Whitney test).

3.2.5 Offspring Effect Index

Different results were found by the %I_O (Table 4), no difference between CAF in scenario 1 and control was observed, and "no effect" classification was recorded. In scenario 2, with CAF exposures, the %I_O presented effect only with 60 mg L^{-1} of CAF, classified in the category "inhibition." On the other hand, an induction effect was evidenced for LAS scenario 1, classified as "evidence of induction." Adversely, scenario 2 with LAS presented an "inhibition" effect.

4 Discussion

Daphnia's EC_{50} for short-time exposure (48 h and 96 h) with CAF in the literature ranges from 177.8

to 445.3 mg L^{-1} of CAF (Chevalier et al., 2015; Di Lorenzo et al., 2019; Lilius et al., 1995; OECD SIDS, 2004). Although CAF is considered a low-risk CEC to aquatic biota, the mixture with other compounds combined with the bioaccumulation capacity in some aquatic species brings attention to the necessity of detailed analysis of its potential environmental hazard (Beasley et al., 2015; Dafouz et al., 2018; Palma et al., 2018). The LAS effect on mobility for *D. magna* acute exposure varies from 0.26 to 55 mg LAS L^{-1} (OECD SIDS, 2005) and depends on the number of carbons in LAS composition and homologs distribution (Prats et al., 1993; van de Plassche et al., 1999; Verge et al., 2001). In our paper, the LAS' negative effect over mobility in the long-term exposure was observed only for scenario 2. The study from van de Plassche et al. (1999) calculated the geometric mean normalized of long-term NOEC for C11.6 LAS in 12 samples and obtained a concentration of 1.4 mg L^{-1} for *D. magna*, explaining why no effect over immobility was observed in scenario 1 for LAS in our study.

Despite recent and constant publications on *D. magna* ecotoxicity tests, few studies have evaluated the CAF and LAS chronic exposure. On the other hand, although our immobility endpoints demonstrate the effects on *D. magna* for the highest concentrations (scenario 2 for LAS and 60 mg L^{-1} of CAF), the reported environmental concentrations (scenario 1) and some tested scenario 2 of CAF (20 and 40 mg L^{-1}) did not present any effect on *D. magna* responses with immobility. Thus, comprehending the effects beyond the classic toxicity endpoints can provide additional support for long-term toxicity studies using LAS and CAF as contaminants (Lewis, 1991).

4.1 Effects on Molting

The scenario 1 for CAF and LAS resulted in no effect on molts produced per parent. However, in scenario 2, the highest CAF concentration presented lower molts produced per parental *Daphnia* and slower intermolt time, resulting in "evidence of inhibition" for $\%I_M$. Besides, the highest LAS concentration in scenario 2 significantly reduced the number of molts and produced an "inhibition" effect for $\%I_M$. Bang et al. (2015) evaluated the *D. magna* survival and reproduction over 21-day exposure to CAF, and no difference in average body length was evidenced for 8.9 mg L^{-1} of CAF compared to the control. Our results demonstrated a decrease in molting numbers for 60 mg L^{-1} of CAF (scenario 2) by $\%I_M$ that can be related to possible inhibition of growth, as seen by Lu et al. (2013) for 10 mg L^{-1} of CAF. These results indicate that the effect over *Daphnia*'s growth is concentration-dependent, reinforced by the slower intermolt time recorded for this CAF concentration, classified as expected effect scenario.

We observed that increasing CAF concentrations led to an improper molting process, since after the release, the old exoskeleton was still attached to the organism's body, affecting *D. magna*'s swimming capability. This release at the end of the molting cycle depends on different enzymes and inorganic ions (Duchet et al., 2011; Reynolds & Samuels, 1996), and the incomplete exoskeleton separation can lead to molting disruption and, in the worst case, organism's death (Song et al., 2017). Few changes in the molting process may not influence overall molting frequency, but, even then, the dysfunctional process confirms that the organism's life is being affected by the toxic compound.

In our study, only 4.9 mg L^{-1} of LAS in scenario 2 presented a statistically distinct effect on molts produced per parent and intermolt time to the control, but with $\%I_M$, a significant variation of molt production was evidenced for all LAS concentrations from scenario 2, showing an inhibition effect. As some statistics could generate false evidence of a biological growth (Lytle, 2001), our result on the molting process endorses the necessity of distinct approaches easily adopted from classical reproduction toxicity tests to detail the ability of *D. magna* confronted by a stressor on the environment. LAS's concentrations with "no effect" classification in $\%I_M$ demonstrated that the difference between average number of molts for the test control and scenario 1 can be assumed as a natural deviation. In this sense, this index confidence range demonstrates lower misestimation of *D. magna* behavior and could reduce false positive or negative effects results when undertaking ecotoxicological evaluation. As any disturbance in the normal molting cycle might increase the Cladocera's sensitivity to the toxicant (Bodar et al., 1990), the inhibition effect in $\%I_M$ was evidenced for concentrations with immobility higher than 80%. Alterations in the molting process can indicate disruption of a multi hormonal system controlled by ecdysteroids (Chang, 1993; Giraudo et al., 2017; LeBlanc, 2007; Mu & LeBlanc, 2002). As no effect was observed in the molts until primiparity for both scenarios with CAF and LAS, we can indicate that the toxic effect over molting needs time to be evidenced.

4.2 Effects on Reproduction

The presence of chemicals in the aquatic environment can affect *D. magna*'s brood quantity and quality (Campos et al., 2016). Overall, the number of offspring produced per parent was more affected by scenario 2 than scenario 1 for both CAF and LAS, which was expected as scenario 2 was described as expected effect concentrations. An $\%I_O$ inhibition effect was recorded for 60 mg L^{-1} of CAF and for all LAS concentrations from scenario 2. Lu et al. (2013) registered a decreasing offspring number per brood with a CAF increase, and with 10 mg L^{-1} of CAF, they observed a 66% reduction in offspring produced per brood per female compared to control. In scenario 1, only 2.5 mg L^{-1} of LAS affected the offspring production, resulting in a higher number than the control. Differences in offspring production may be related not only to the type of contaminant but also to its concentration and increase in the offspring produced per parent causes changes in the life cycle of *D. magna* and can impact the dynamics of aquatic ecosystems, producing a negative effect on the population (Cleuvers et al., 1997; Goser & Ratte, 1994; Preuss et al., 2009). In our experiments, the results suggested that environmental concentrations of CAF did not increase the offspring produced but induced a fast brood release.

The offspring produced depended directly on LAS concentration and presented two distinct effects, an $\%I_O$ induction was evidenced for scenario 1 concentrations and $\%I_O$ inhibition occurred in scenario 2 concentration. A previous study has indicated that LAS presents low hazard effects under detected environmental concentrations, presenting chronic NOECs from 1.2 to 3.2 mg L^{-1} (Taylor, 1985). The results found in this paper indicated that the environmental contamination of LAS could increase *D. magna* population. However, this could lead to an overcrowding effect, negatively impacting aquatic ecosystem dynamics over the population (Cleuvers et al., 1997; Goser & Ratte, 1994; Preuss et al., 2009). Our data demonstrate the necessity of analyzing different effects of ecological importance in classic toxicity tests, such as alteration of reproductive strategy and change in growth rate (Hayes et al., 2002). Ecologically, the presence of a chemical can affect both the quantity and quality of the offspring produced (Campos et al., 2016), and through $\%I_O$ index, we evidenced the distinct effect of LAS over the quantity of offspring produced. Therefore, these differences are not only dependent on the chemical type but vary with LAS concentrations.

4.3 Relation Between Molts Number and Offspring Production

During the organism's natural life cycle, neonates are released during ecdysis and, after the molting, another set of oocytes begins to move to the maturation phase in the incubator chamber; therefore, it would be expected that the acceleration in the ecdysis process is accompanied by the induction of neonates production (Dodson et al., 2010). However, in our study, there was no evidence of molting induction when offspring induction was recorded (scenario 1 for LAS). We can suggest that, from the relationship between the rate of neonates' production and the process of ecdysis, the organism allocates energy primarily for the species' perpetuation (Dodson et al., 2010).

Different pollutants show contrasting dominant ecotoxicological modes of action (Barata & Baird, 2000), and from the $\%I_M$ and $\%I_O$ indexes, it is possible to compare different reproductive strategies of *D. magna*. In scenario 1, daphnids did not alter their molt production, but increased their offspring with LAS. Environmental contamination can result in changes in daphnids' density, growth, and reproduction. As this species feeds on primary producers (e.g., algae) and serves as food for final consumers (e.g., fish), these changes may cause disequilibrium in this population level and consequently in the food chain (Tanaka et al., 2018), highlighting the importance of studying the individual's growth and reproduction effects (Bruijning et al., 2018). Moreover, with the constant increase usages of the CAF and LAS worldwide (Badmus et al., 2021; Quadra et al., 2020), its environmental concentrations are expected to increase, and scenario 2 could represent real scenario, demonstrating how important is to evaluate these scenarios in our paper.

The complementary indexes proposed in this paper ($\%I_M$ and $\%I_O$) were suitable for evaluating induction and inhibition effects with LAS and CAF and can complement *D. magna* classic toxicological analysis for lethal and sublethal conditions. Moreover, the main advantage of applying these suggested indexes is to deduce any distinct behavior by observing the organism's development. The daily analysis allows the verification of the swimming trend and how the organism uses its energy (e.g., growth or reproduction), and complements the indexes' interpretation.

5 Conclusion

LAS and CAF were used in this paper for analyzing and comprehending the toxic effects on molting and reproduction process in long-term exposures. According to our findings, the indexes $\%I_M$ and $\%I_O$ were comparable to other endpoints in two scenarios and can add information on *D. magna* ecotoxicological analysis. In the same assay, we have results of classical endpoints, and we can qualify the information about the molting and reproduction process by calculating the $\%I_M$ and $\%I_O$ indexes. From the results, we can indicate that LAS and CAF produced contrasting dominant ecotoxicological responses. From the $\%I_M$ and $\%I_O$ data, it is possible to verify that LAS produces no effect on the molting process and an "induction" effect on offspring production in reported environmental concentrations (scenario 1). Moreover, in scenario 2, LAS produced an "inhibition" effect over $\%I_O$ and "evidence of inhibition" and an "inhibition" effect on the *D. magna* molting production. CAF results indicated that in expected environmental concentrations, scenario 1, no effect was observed in both indexes. Our results indicated that CAF inhibited more offspring than molting for 60 mg L^{-1} of CAF. This complementary information on the *Daphnia* life cycle with LAS and CAF demonstrated this approach to classic toxicological data for environmentally relevant pollutants. Further use of these indexes with distinct substances can improve information on ecotoxicological assessments.

Author Contribution Mara R. de Lima e Silva, Mayara C. Felipe, and Aline C. Bernegossi conceived and designed experiments. Mara R. de Lima e Silva, Mayara C. Felipe, and Aline C. Bernegossi performed experiments. Aline C. Bernegossi and Gleyson B. Castro performed the statistical analysis. Mara R. de Lima e Silva performed the index calculation. Mara R. de Lima e Silva, Aline C. Bernegossi, Gleyson B. Castro, Allan P. Ogura, and Mayara C. Felipe wrote the manuscript. Mara R. de Lima e Silva and Juliano J. Corbi provided technical and editorial assistance.

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Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest I, Mara R. de Lima e Silva, declare that the authorship and content of the manuscript "*Assessing caffeine and LAS effects on molting and reproduction of Daphnia magna by quantitative and qualitative approaches*" has been approved by all authors, and prevailing that all local, national, and international conventions and regulations, and the normal scientific ethical practices have been respected. The authors of this study attribute rights to the Water, Air, & Soil Pollution journal where this is published. We ask that academics have rights to post and use this paper. In view of this I declare no conflict of interest among the authors in this paper or between the Institutions involved in this research.

References

ABNT - Associação Brasileira de Normas Técnicas. (2011). *NBR 12648 — Ecotoxicologia aquática — Toxicidade crônica — Método de ensaio com algas (Chlorophyceae)* (Vol. 12648). Rio de Janeiro.

ABNT - Associação Brasileira de Normas Técnicas. (2016). NBR 15499 Ecotoxicologia aquática — Toxicidade crônica de curta duração — Método de ensaio com peixes, 1–29.

Altenburger, R., Brack, W., Burgess, R. M., Busch, W., Escher, B. I., Focks, A., et al. (2019). Future water quality monitoring: Improving the balance between exposure and toxicity assessments of real-world pollutant mixtures. *Environmental Sciences Europe, 31*(1), 12. https://doi.org/10.1186/s12302-019-0193-1

Anderson, B. G. (1944). The toxicity thresholds of various substances found in industrial wastes as wetermined by the use of Daphnia magna. *Sewage Works Journal, 16*(6), 1156–1165. http://www.jstor.org/stable/25029937.

Anderson, B. G., & Jenkins, J. C. (1942). A time study of events in the life span of Daphnia magna. *Biological Bulletin, 83*(2), 18. https://books.google.ca/books?id=GEqpngEACAAJ.

Atici, A. A. (2021). Seasonal changes of some detergent components in surface water of rivers to the Van Lake, Turkey. *Toxicological & Environmental Chemistry*, 1–10.https://doi.org/10.1080/02772248.2021.1882460

Badmus, S. O., Amusa, H. K., Oyehan, T. A., & Saleh, T. A. (2021). Environmental risks and toxicity of surfactants: Overview of analysis, assessment, and remediation techniques. *Environmental Science and Pollution Research, 28*(44), 62085–62104. https://doi.org/10.1007/s11356-021-16483-w

Bang, S. H., Ahn, J.-Y., Hong, N.-H., Sekhon, S. S., Kim, Y.-H., & Min, J. (2015). Acute and chronic toxicity assessment and the gene expression of Dhb, Vtg, Arnt, CYP4, and CYP314 in Daphnia magna exposed to pharmaceuticals. *Molecular & Cellular Toxicology, 11*(2), 153–160. https://doi.org/10.1007/s13273-015-0013-7

Barata, C., & Baird, D. J. (2000). Determining the ecotoxicological mode of action of chemicals from measurements made on individuals: Results from instar-based tests with Daphnia magna Straus. *Aquatic Toxicology, 48*(2–3), 195–209. https://doi.org/10.1016/S0166-445X(99)00038-7

Beasley, A., Belanger, S. E., Brill, J. L., & Otter, R. R. (2015). Evaluation and comparison of the relationship between NOEC and EC10 or EC20 values in chronic Daphnia toxicity testing. *Environmental Toxicology and Chemistry, 34*(10), 2378–2384. https://doi.org/10.1002/etc.3086

Benotti, M. J., & Brownawell, B. J. (2009). Microbial degradation of pharmaceuticals in estuarine and coastal seawater. *Environmental Pollution, 157*(3), 994–1002. https://doi.org/10.1016/j.envpol.2008.10.009

Bodar, C. W. M., Voogt, P. A., & Zandee, D. I. (1990). Ecdysteroids in Daphnia magna: Their role in moulting and reproduction and their levels upon exposure to cadmium. *Aquatic Toxicology, 17*(4), 339–350. https://doi.org/10.1016/0166-445X(90)90016-I

Brack, W., Dulio, V., & Slobodnik, J. (2012). The NORMAN Network and its activities on emerging environmental substances with a focus on effect-directed analysis of complex environmental contamination. *Environmental Sciences Europe, 24*(1), 29. https://doi.org/10.1186/2190-4715-24-29

Bruijning, M., ten Berge, A. C. M., & Jongejans, E. (2018). Population-level responses to temperature, density and clonal differences in Daphnia magna as revealed by integral projection modelling. *Functional Ecology, 32*(10), 2407–2422. https://doi.org/10.1111/1365-2435.13192

Buerge, I. J., Poiger, T., Mller, M. D., & Buser, H. (2006). Combined sewer overflows to surface waters detected by the anthropogenic marker caffeine. *Environmental Science & Technology, 40*(13), 4096–4102. https://doi.org/10.1021/es052553l

Busse, L., & Nagoda, C. (2015). *Detection of caffeine in the streams and rivers within the San Diego region—Pilot study*. Surface Water Monitoring Program.

Campos, B., Jordão, R., Rivetti, C., Lemos, M. F. L., Soares, A. M. V. M., Tauler, R., & Barata, C. (2016). Two-generational effects of contaminants in Daphnia magna: Effects of offspring quality. *Environmental Toxicology and Chemistry, 35*(6), 1470–1477. https://doi.org/10.1002/etc.3290

Cantwell, M. G., Katz, D. R., Sullivan, J. C., Borci, T., & Chen, R. F. (2016). Caffeine in Boston Harbor past and present, assessing its utility as a tracer of wastewater contamination in an urban estuary. *Marine Pollution Bulletin, 108*(1–2), 321–324. https://doi.org/10.1016/j.marpolbul.2016.04.006

Chang, E. S. (1993). Comparative endocrinology of molting and reproduction: Insects and crustaceans. *Annual Review of Entomology, 38*(1), 161–180.

Chen, Z., Pavelic, P., Dillon, P., & Naidu, R. (2002). Determination of caffeine as a tracer of sewage effluent in natural waters by on-line solid-phase extraction and liquid chromatography with diode-array detection. *Water Research, 36*(19), 4830–4838. https://doi.org/10.1016/S0043-1354(02)00221-X

Chevalier, J., Harscoët, E., Keller, M., Pandard, P., Cachot, J., & Grote, M. (2015). Exploration of Daphnia behavioral effect profiles induced by a broad range of toxicants with different modes of action. *Environmental Toxicology and Chemistry, 34*(8), 1760–1769. https://doi.org/10.1002/etc.2979

Cleuvers, M., Goser, B., & Ratte, H.-T. (1997). Life-strategy shift by intraspecific interaction in Daphnia magna: Change in reproduction from quantity to quality. *Oecologia, 110*(3), 337–345. https://doi.org/10.1007/s004420050167

Corbi, J. J., Trivinho-Strixino, S., & dos Santos, A. (2008). Environmental evaluation of metals in sediments and dragonflies due to sugar cane cultivation in neotropical streams. *Water, Air, and Soil Pollution, 195*(1–4), 325–333. https://doi.org/10.1007/s11270-008-9749-1

Dafouz, R., Cáceres, N., Rodríguez-Gil, J. L., Mastroianni, N., López de Alda, M., Barceló, D., et al. (2018). Does the presence of caffeine in the marine environment represent an environmental risk? A regional and global study. *Science of the Total Environment, 615*, 632–642. https://doi.org/10.1016/j.scitotenv.2017.09.155

Daneshvar, A., Aboulfadl, K., Viglino, L., Broséus, R., Sauvé, S., Madoux-Humery, A. S., et al. (2012). Evaluating pharmaceuticals and caffeine as indicators of fecal contamination in drinking water sources of the Greater Montreal region. *Chemosphere, 88*(1), 131–139. https://doi.org/10.1016/j.chemosphere.2012.03.016

Di Lorenzo, T., Castaño-Sánchez, A., Di Marzio, W. D., García-Doncel, P., Nozal Martínez, L., Galassi, D. M. P., & Iepure, S. (2019). The role of freshwater copepods in the environmental risk assessment of caffeine and propranolol mixtures in the surface water bodies of Spain. *Chemosphere, 220*, 227–236. https://doi.org/10.1016/J.CHEMOSPHERE.2018.12.117

Dodson, S. L., Cáceres, C. E., & Rogers, D. C. (2010). Cladocera and other Branchiopoda. *Ecology and classification of North American freshwater invertebrates* (3rd ed., Vol. 66, pp. 1008–1013). Elsevier Ltd.

Duchet, C., Mitie Inafuku, M., Caquet, T., Larroque, M., Franquet, E., Lagneau, C., & Lagadic, L. (2011). Chitobiase activity as an indicator of altered survival, growth and reproduction in Daphnia pulex and Daphnia magna (Crustacea: Cladocera) exposed to spinosad and diflubenzuron.

Ecotoxicology and Environmental Safety, 74(4), 800–810. https://doi.org/10.1016/j.ecoenv.2010.11.001

Edwards, Q. A., Kulikov, S. M., & Garner-O'Neale, L. D. (2015). Caffeine in surface and wastewaters in Barbados, West Indies. *Springerplus, 4*(1), 57. https://doi.org/10.1186/s40064-015-0809-x

Eichhorn, P., Rodrigues, S. V., Baumann, W., & Knepper, T. P. (2002). Incomplete degradation of linear alkylbenzene sulfonate surfactants in Brazilian surface waters and pursuit of their polar metabolites in drinking waters. *Science of the Total Environment, 284*(1–3), 123–134. https://doi.org/10.1016/S0048-9697(01)00873-7

Fox, K., Holt, M., Daniel, M., Buckland, H., & Guymer, I. (2000). Removal of linear alkylbenzene sulfonate from a small Yorkshire stream: Contribution to GREAT-ER project #7. *Science of the Total Environment, 251–252*, 265–275. https://doi.org/10.1016/S0048-9697(00)00389-2

Giraudo, M., Douville, M., Cottin, G., & Houde, M. (2017). Transcriptomic, cellular and life-history responses of Daphnia magna chronically exposed to benzotriazoles: Endocrine-disrupting potential and molting effects. *PLoS ONE, 12*(2), e0171763. https://doi.org/10.1371/journal.pone.0171763

Gonçalves, E. S., Rodrigues, S. V., & da Silva-Filho, E. V. (2017). The use of caffeine as a chemical marker of domestic wastewater contamination in surface waters: Seasonal and spatial variations in Teresópolis, Brazil. *Ambiente e Agua—An interdisciplinary Journal of Applied Science, 12*(2), 192. https://doi.org/10.4136/ambi-agua.1974

Goser, B., & Ratte, H. T. (1994). Experimental evidence of negative interference in Daphnia magna. *Oecologia, 98*(3–4), 354–361. https://doi.org/10.1007/BF00324224

Hampel, M., Mauffret, A., Pazdro, K., & Blasco, J. (2012). Anionic surfactant linear alkylbenzene sulfonates (LAS) in sediments from the Gulf of Gdańsk (southern Baltic Sea, Poland) and its environmental implications. *Environmental Monitoring and Assessment, 184*(10), 6013–6023. https://doi.org/10.1007/s10661-011-2399-6

Hayes, T. B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A. A., & Vonk, A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proceedings of the National Academy of Sciences, 99*(8), 5476–5480. https://doi.org/10.1073/pnas.082121499

Holt, M. S., Fox, K. K., Burford, M., Daniel, M., & Buckland, H. (1998). UK monitoring study on the removal of linear alkylbenzene sulphonate in trickling filter type sewage treatment plants. Contribution to GREAT-ER project # 2. *Science of the Total Environment, 210–211*, 255–269. https://doi.org/10.1016/S0048-9697(98)00016-3

Hoogenboom, L. A. P., Hamers, A. R. M., & Bovee, T. F. H. (1999). Bioassays for the detection of growth-promoting agents, veterinary drugs and environmental contaminants in food. *The Analyst, 124*(1), 79–85. https://doi.org/10.1039/a804950e

Hu, X., Ren, C., Kang, W., et al. (2018). Characterization and toxicity of nanoscale fragments in wastewater treatment plant effluent. *Science of the Total Environment, 626*, 1332–1341. https://doi.org/10.1016/j.scitotenv.2018.01.180

INCTAA - Instituto Nacional de Ciências e Tecnologias Analíticas Avançadas. (2014). *Cafeína em águas de abastecimento público no Brasil*. (M. C. Canela, Ed.). São Carlos - Brazil: Editora Cubo.

Jagoda, A., Żukowski, W., & Dąbrowska, B. (2015). Investigations of the presence of caffeine in the Rudawa River, Kraków, Poland. *Environmental Monitoring and Assessment*, *187*(9). https://doi.org/10.1007/s10661-015-4760-7

Knepper, T. P., Barceló, D., & de Voogt, P. (2003). *Analysis and fate of surfactants in the aquatic environment*. Elsevier.

Kolpin, D. W., & Meyer, M. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U. S. Streams, 1999–2000: A national reconnaissance. *Environmental Science & Technology, 36*(6), 1202–1211. https://doi.org/10.1021/es011055j

LeBlanc, G. A. (2007). Crustacean endocrine toxicology: A review. *Ecotoxicology, 16*(1), 61–81.

Lewis, M. A. (1991). Chronic and sublethal toxicities of surfactants to aquatic animals: A review and risk assessment. *Water Research, 25*(1), 101–113. https://doi.org/10.1016/0043-1354(91)90105-Y

Lilius, H., Hästbacka, T., & Isomaa, B. (1995). Short Communication: A comparison of the toxicity of 30 reference chemicals to Daphnia Magna and Daphnia Pulex. *Environmental Toxicology and Chemistry, 14*(12), 2085–2088. https://doi.org/10.1002/etc.5620141211

Lu, G., Li, Z., & Liu, J. (2013). Effects of selected pharmaceuticals on growth, reproduction and feeding of Daphnia magna. *Fresenius Environmental Bulletin, 22*(09), 2588–2594.

Luo, Y., Guo, W., Ngo, H. H., et al. (2014). A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Science of the Total Environment, 473–474*, 619–641. https://doi.org/10.1016/j.scitotenv.2013.12.065

Lytle, D. A. (2001). Convergent growth regulation in arthropods: Biological fact or statistical artifact? *Oecologia, 128*(1), 56–61. https://doi.org/10.1007/s004420100639

Machado, K. C., Grassi, M. T., Vidal, C., Pescara, I. C., Jardim, W. F., Fernandes, A. N., et al. (2016). A preliminary nationwide survey of the presence of emerging contaminants in drinking and source waters in Brazil. *Science of the Total Environment, 572*, 138–146. https://doi.org/10.1016/j.scitotenv.2016.07.210

Martin-Creuzburg, D., Westerlund, S. A., & Hoffmann, K. H. (2007). Ecdysteroid levels in Daphnia magna during a molt cycle: Determination by radioimmunoassay (RIA) and liquid chromatography–mass spectrometry (LC–MS). *General and Comparative Endocrinology, 151*(1), 66–71. https://doi.org/10.1016/j.ygcen.2006.11.015

Montagner, C. C., & Jardim, W. F. (2011). Spatial and seasonal variations of pharmaceuticals and endocrine disruptors in the Atibaia River, São Paulo State (Brazil). *Journal of the Brazilian Chemical Society, 22*(8), 1452–1462. https://doi.org/10.1590/S0103-50532011000800008

Moore, M. T., Greenway, S. L., Farris, J. L., & Guerra, B. (2008). Assessing caffeine as an emerging environmental concern using conventional approaches. *Archives of Environmental Contamination and Toxicology, 54*(1), 31–35. https://doi.org/10.1007/s00244-007-9059-4

Mu, X., & LeBlanc, G. A. (2002). Environmental antiecdysteroids alter embryo development in the crustacean Daphnia magna. *Journal of Experimental Zoology, 292*(3), 287–292.

NORMAN - Network of reference laboratories and related organisations for monitoring and bio-monitoring of emerging environmental substances. (2016). *NORMAN prioritisation framework for emerging substances*. www.norman-network.net.

OECD SIDS - Organisation for Economic Co-operation and Development and Development Screening Information Data Sets. (2004). *Screening Information Data Sets - Caffeine*.

OECD SIDS- Organisation for Economic Co-operation and Development. (2005). Linear alkylbenzene sulfonate (LAS). *SIDS Initial Assessment Report*, 1–357.

OECD US - Emerging Contaminants Workgroup. (2008). *White paper aquatic life criteria for contaminants of emerging concern part I general challenges and recommendations prepared by the OW/ORD Emerging Contaminants Workgroup notice this document is an internal planning document*.

OECD - Organisation for Economic Co-operation and Development. (2012). *Test no. 211: Daphnia magna reproduction test*. OECD. https://doi.org/10.1787/9789264185203-en.

Olkova, A. S., Kantor, G. Y., Kutyavina, T. I., & Ashikhmina, T. Y. (2017). The importance of maintenance conditions of Daphnia magna Straus as a test organism for ecotoxicological analysis. *Environmental Toxicology and Chemistry, 37*(2), 376–384. https://doi.org/10.1002/etc.3956

Palma, D., Bianco Prevot, A., Brigante, M., Fabbri, D., Magnacca, G., Richard, C., et al. (2018). New insights on the photodegradation of caffeine in the presence of bio-based substances-magnetic iron oxide hybrid nanomaterials. *Materials, 11*(7), 1084. https://doi.org/10.3390/ma11071084

Prats, D., Ruiz, F., Vazquez, B., Zarzo, D., Berna, J. L., Moreno, A., et al. (1993). LAS homolog distribution shift during wastewater treatment and composting: Ecological implications. *Environmental Toxicology and Chemistry, 12*(9), 1599–1608. https://doi.org/10.1002/etc.5620120908

Preuss, T. G., Hammers-Wirtz, M., Hommen, U., Rubach, M. N., & Ratte, H. T. (2009). Development and validation of an individual based Daphnia magna population model: The influence of crowding on population dynamics. *Ecological Modelling, 220*(3), 310–329. https://doi.org/10.1016/j.ecolmodel.2008.09.018

Quadra, G. R., Paranaíba, J. R., Vilas-Boas, J., Roland, F., Amado, A. M., Barros, N., et al. (2020). A global trend of caffeine consumption over time and related-environmental impacts. *Environmental Pollution, 256*, 113343. https://doi.org/10.1016/j.envpol.2019.113343

R Core Team. (2018). R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. https://www.r-project.org.

Reddy, V. R., & Behera, B. (2006). Impact of water pollution on rural communities: An economic analysis. *Ecological Economics, 58*(3), 520–537. https://doi.org/10.1016/j.ecolecon.2005.07.025

Reynolds, S. E., & Samuels, R. I. (1996). Physiology and biochemistry of insect moulting fluid. *Advances in Insect Physiology, 26*(C), 157–232. https://doi.org/10.1016/S0065-2806(08)60031-4

Sanderson, H., Dyer, S. D., Price, B. B., Nielsen, A. M., van Compernolle, R., Selby, M., et al. (2006). Occurrence and weight-of-evidence risk assessment of alkyl sulfates, alkyl ethoxysulfates, and linear alkylbenzene sulfonates (LAS) in river water and sediments. *Science of the Total Environment, 368*(2–3), 695–712. https://doi.org/10.1016/J.SCITOTENV.2006.04.030

Sauvé, S., & Desrosiers, M. (2014). A review of what is an emerging contaminant. *Chemistry Central Journal, 8*, 15. https://doi.org/10.1186/1752-153X-8-15

Schaider, L. A., Swetschinski, L., Campbell, C., & Rudel, R. A. (2019). Environmental justice and drinking water quality: Are there socioeconomic disparities in nitrate levels in U.S. drinking water? *Environmental Health, 18*(1), 3. https://doi.org/10.1186/s12940-018-0442-6

Sierra-Alvarez, R., & Lettinga, G. (1991). The effect of aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge. *Applied Microbiology and Biotechnology, 34*(4), 544–550. https://doi.org/10.1007/BF00180586

Silva, C. P., Lima, D. L. D., Schneider, R. J., Otero, M., & Esteves, V. I. (2014). Evaluation of the anthropogenic input of caffeine in surface waters of the north and center of portugal by ELISA. *Science of the Total Environment, 479–480*(1). https://doi.org/10.1016/j.scitotenv.2014.01.120

Snyder, S. A., Wert, E. C., Lei, H. D., Westerhoff, P., & Yoon, Y. (2007). Removal of EDCs and pharmaceuticals in drinking and reuse treatment processes. *American Water Works Association Research Foundation Report*, 331.

Song, Y., Villeneuve, D. L., Toyota, K., Iguchi, T., & Tollefsen, K. E. (2017). Ecdysone receptor agonism leading to lethal molting disruption in arthropods: Review and adverse outcome pathway development. *Environmental Science and Technology*. American Chemical Society. https://doi.org/10.1021/acs.est.7b00480

Sposito, J. C., Montagner, C., Casado, M., Navarro-Martín, L., Solórzano, J. C., Piña, B., & Grisolia, A. B. (2018). Emerging contaminants on Brazilian rivers: Ocurrence and effects on gene expression in zebrafish (*Danio rerio*) embryos. *Chemosphere, 209*, 696–704.

Struewing, K. A., Lazorchak, J. M., Weaver, P. C., Johnson, B. R., Funk, D. H., & Buchwalter, D. B. (2015). Part 2: Sensitivity comparisons of the mayfly Centroptilum triangulifer to Ceriodaphnia dubia and Daphnia magna using standard reference toxicants; NaCl, KCl and CuSO4. *Chemosphere, 139*, 597–603. https://doi.org/10.1016/j.chemosphere.2014.04.096

Sui, Q., Huang, J., Deng, S., Yu, G., & Fan, Q. (2010). Occurrence and removal of pharmaceuticals, caffeine and DEET in wastewater treatment plants of Beijing, China. *Water Research, 44*(2), 417–426. https://doi.org/10.1016/j.watres.2009.07.010

Sumiya, E., Ogino, Y., Miyakawa, H., Hiruta, C., Toyota, K., Miyagawa, S., & Iguchi, T. (2014). Roles of ecdysteroids for progression of reproductive cycle in the fresh water

crustacean Daphnia magna. *Frontiers in Zoology, 11*(1), 60. https://doi.org/10.1186/s12983-014-0060-2
Tanaka, Y., Nakamura, K., Oda, S., Watanabe, H., & Tatarazako, N. (2018). Estimation of population-level effect of the endocrine disruptor pyriproxyfen in Daphnia magna by using changes in sex ratio and reproductive output. *Ecotoxicology and Environmental Safety, 156*, 463–475. https://doi.org/10.1016/j.ecoenv.2018.03.044
Taylor, M. (1985). Effect of diet on the sensitivity of Daphnia magna to linear alkylbenzene sulfonate. In R. Purdy, R. D. Cardwell, & R. Comotto Bahner (Eds.), *Aquatic toxicology and hazard assessment: Seventh symposium* (pp. 53-53–20). ASTM International.
Temple, J. L., Bernard, C., Lipshultz, S. E., Czachor, J. D., Westphal, J. A., & Mestre, M. A. (2017). The safety of ingested caffeine: A comprehensive review. *Frontiers in Psychiatry*, *8*.https://doi.org/10.3389/fpsyt.2017.00080
van de Plassche, E. J., de Bruijn, J. H. M., Stephenson, R. R., Marshall, S. J., Feijtel, T. C. J., & Belanger, S. E. (1999). Predicted no-effect concentrations and risk characterization of four surfactants: Linear alkyl benzene sulfonate, alcohol ethoxylates, alcohol ethoxylated sulfates, and soap. *Environmental Toxicology and Chemistry, 18*(11), 2653–2663. https://doi.org/10.1002/etc.5620181135
Verge, C., Moreno, A., Bravo, J., & Berna, J. L. (2001). Influence of water hardness on the bioavailability and toxicity of linear alkylbenzene sulphonate (LAS). *Chemosphere, 44*(8), 1749–1757. https://doi.org/10.1016/S0045-6535(00)00574-9
Wang, C., Shi, H., Adams, C. D., Gamagedara, S., Stayton, I., Timmons, T., & Ma, Y. (2011). Investigation of pharmaceuticals in Missouri natural and drinking water using high performance liquid chromatography-tandem mass spectrometry. *Water Research, 45*(4), 1818–1828. https://doi.org/10.1016/j.watres.2010.11.043
You, L., Nguyen, V. T., Pal, A., Chen, H., He, Y., Reinhard, M., & Gin, K. Y. H. (2015). Investigation of pharmaceuticals, personal care products and endocrine disrupting chemicals in a tropical urban catchment and the influence of environmental factors. *Science of the Total Environment*, *536*.https://doi.org/10.1016/j.scitotenv.2015.06.041
Zhu, Y.-P., Rosen, M. J., Morrall, S. W., & Tolls, J. (1998). Surface properties of linear alkyl benzene sulfonates in hard river water. *Journal of Surfactants and Detergents, 1*(2), 187–193. https://doi.org/10.1007/s11743-998-0018-2