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Mortality and Effect on Growth of *Artemia franciscana* Exposed to Two Common Organic Pollutants

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Abstract: Acute toxicity and inhibition on growth of *Artemia franciscana* nauplii (Instar I-II) after exposure to the reference toxicants bisphenol a (BPA) and sodium dodecyl sulfate (SDS) were studied. LC_{50} values were calculated and differences in body growth were recorded after 24, 48, and 72 h of exposure to the toxicants. The results indicated that BPA had lower toxicity than SDS. Development of the nauplii was clearly influenced by duration of exposure. Growth inhibition was detected for both toxicants. Abnormal growth of the central eye of several *Artemia* nauplii after 72 h of exposure to BPA was also detected. Our results indicate that growth inhibition could be used as a valid endpoint for toxicity studies.

Keywords: acute toxicity; sodium dodecyl sulfate; bisphenol a; bioassays; LC_{50}; probit analysis

1. Introduction

The Water Framework Directive (WFD) is an ambitious and promising European legislative tool aiming to achieve good water quality in all European waters by 2027 [1]. It is even considered by some as the most significant international legislation in the field of Water Quality that was introduced for many years [2,3]. Monitoring of water contamination serves as a cornerstone for the implementation of WFD, which, in turn, requires the development and constant improvement of an array of in vitro, in vivo, and in situ bioanalytical methods to identify pollutants and assess their effects [4]. While chemical analyses of water bodies for detection of pollutants and estimation of contamination levels was the most common practice, it is no longer considered sufficient for water quality control, but must be complemented by ecotoxicological end points [5]. Therefore, in that context, biomonitoring tests can be essential tools in fulfilling the WFD requirements [2], mainly due to their ability to quickly deliver reliable data at an affordable cost [6].

Aquatic invertebrates could be that essential tool, since they can guarantee the reliability, feasibility, and cost-effectiveness in routine and/or research ecotoxicity practices [7]. As test subjects, they are relatively easy to maintain in laboratory conditions, their use involves fewer ethical considerations, doses are easier to deliver in the aquatic medium, and their small size and inexpensive cultural requirements allow larger data sets to be collected [8]. Several marine crustaceans have been proposed as potential candidates to investigate the biological effects of contaminants and environmental matrices on primary consumers, by utilizing their larval stages in bioassays [9], with brine shrimps (*Artemia* spp.) being one of the most frequently used species for toxicity testing [10–14].

One of the most easily measured responses of organisms to environmental conditions is their somatic growth, since it is influenced by availability and quality of food, temperature, water chemistry, biotic factors (e.g., competitors, diseases, parasites), and other environmental stressors, including pollutants [15]. Growth inhibition can be considered as a teratogen test system based on the disturbance of elongation development, relative to controls raised at the same time and conditions [14]. Contrary to other invertebrates used in toxicity testing, newly hatched *Artemia* nauplii do not require feeding for the first 72 h [16], so any changes in growth during a bioassay could be safely attributed to the pollutant. Still, despite the clear advantages, teratogenicity is among the less popular studied endpoints of *Artemia* spp., with only just a few papers and citations [11,14,17]. The aim of this study was to determine the acute toxicity effects, in terms of mortality and growth inhibition, of two reference toxicants, which include bisphenol-a (BPA) and sodium dodecyl sulfate (SDS), on *Artemia franciscana* nauplii after 24, 48, and 72 h of exposure.

These specific substances were selected because they both are among the most commonly produced and used chemicals in the world [18,19]. BPA, on the one hand, is used in the synthesis of polycarbonates, epoxy resins, and thermal paper, which commonly appears in various products for everyday use including water-pipes, electronic equipment, paper, or toys [20]. BPA is also used in food contact materials including packaging, bottles, and lacquer coatings for tins leading to exposure of consumers to BPA through food and drinking water [19]. SDS, on the other hand, is a widely employed detergent, with applications in household products, industrial mixtures, and cleansing products in cosmetics. It is also found in liquid soaps and shampoos, bubble baths, bath and shower gels, and toothpaste [18]. Both substances have become ubiquitous in the environment [20] and are now considered as common environmental contaminants, which might have strong implications towards the ecosystem health [8,18].

2. Materials and Methods

2.1. Preparation of Solutions and Hatching of Artemia

Synthetic sea water (SSW) at 35 psu was used as a negative control, as a solvent for the preparation of stock solutions, and as a diluent for the testing solutions. It was prepared by dissolving the marine salt mixture Instant Ocean® in deionized water and was, afterward, stored and aerated at room temperature for approximately 24 h before the analyses. Prior to use, it was filtered through a Whatman Grade 589/3 filter paper (<2 μm) and then tested to verify that salinity was maintained at 35 psu and oxygen content was maintained at >90% saturation [10]. Fresh standard 200 ppm stock solutions of SDS (Sigma Aldrich, Munich, Germany ≥99%, CAS# 151-21-3) and BPA (Sigma Aldrich, Munich, Germany ≥99%, CAS# 80-05-7) were prepared by dissolving the substances in SSW, a few hours before analyses. For each toxicity test, stock solutions were diluted in SSW in order to obtain the required concentrations for each level of exposure.

Larvae of brine shrimp, *A. franciscana*, were hatched from dehydrated cysts of the previously characterized strain (AF/F2006, Laboratory for Biological Research in Aquatic Pollution of Ghent University, Belgium). The hatching procedure was initiated approximately 24 h before the beginning of each test. SSW was used as the hydration medium and the cysts were hydrated under standard established conditions [10,12].

2.2. Acute Mortality Tests

Before the actual toxicity tests, a "preliminary" test was performed (data not shown), in order to determine the "critical range" for each toxicant and exposure time [10]. For estimating LC_{50} of SDS, the solutions used were 7, 8, 9, 10, and 12 ppm for all three exposure periods (24, 48, and 72 h). On the contrary, for BPA, different solutions were used for each period: 40, 42, 44, 46, and 48 ppm for 24 h of exposure, 32, 34, 36, 38, and 40 ppm for 48 h of exposure, and 15, 17, 19, 21, and 23 ppm for 72 h of exposure.

Acute mortality tests were performed in multi-well plates (4 × 6 wells, Greiner CELLSTAR®). In each well/replicate, 1 mL of each solution was placed and 10 *Artemia* nauplii (Instar I–II) were transferred with a micropipette, whereas control wells contained only SSW and nauplii. A total of 9 replicates were used for each concentration/exposure combination (3 replicates per plate × 3 plates for each exposure). The multi-well plates were covered with parafilm, in order to minimize evaporation of the toxic compounds and were kept at 25 °C in the dark [10].

After 24, 48, and 72 h of exposure, larvae that were completely motionless were considered to be dead, and the percentage of mortality was calculated when compared to the control. The term "motionless" means organisms that do not change their own barycenter position and do not move any appendages within 10 to 15 s [9,14]. Tests were considered valid when control mortality did not exceed 10% [10].

2.3. Effect on Growth

To estimate the effects on growth, 500 *Artemia* nauplii (Instar I–II) were cultured in 500 mL conical flasks and exposed in a series of lethal and sublethal concentrations, which are 4, 5, 6, 7, 8, 9, 10, and 12 ppm for SDS and 3, 5, 8, 10, 15, 20, 25, and 30 ppm for BPA. Each flask contained a solution from each concentration, whereas the control flask contained only SSW. Tests were performed in triplicate, adding to a total of 27 flasks for each toxicant (9 concentrations × 3 replicates). Cultures were developed under stable conditions (T = 24–26 °C, Salinity = 35 psu, continuous oxygen supply, and illumination of 3000 to 4000 lux).

From each flask, 50 individuals were randomly collected at 24, 48. 72 h of exposure, they were placed in a petri dish under an Olympus SZX-9 stereomicroscope connected with an Infinity-1 microscopy camera (Teledyne Lumenera, Ottawa, Canada) and were photographed in triplets. Digital photos were used in order to measure the body length of the larvae to the nearest 1 mm, by measuring the length between the well-pigmented eye and the poorly defined end of the tail [14] (Figure 1) but also to identify any other potential malformations. Processing of the digital photos was performed using the software analySIS getIT 5.1 (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

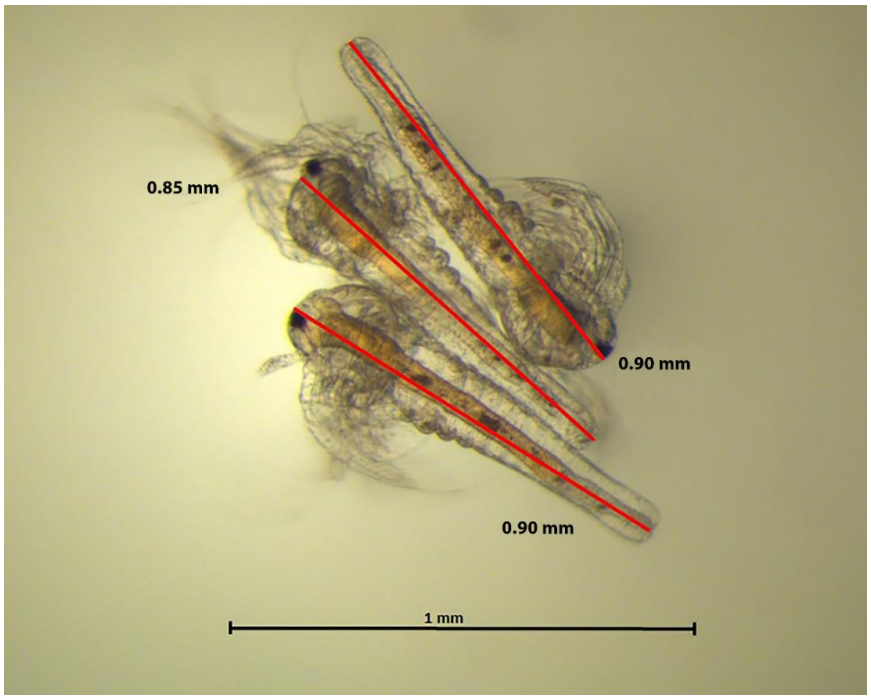

Figure 1. Body length measurement of *Artemia franciscana* nauplii.

2.4. Stability of Compounds

Since SDS is a rather stable compound [21], any slight changes in the concentration of the different solutions during tests were considered negligible. On the contrary, BPA is biodegraded in the environment and can undergo direct photolysis [22]. Therefore, in order to investigate the stability of BPA in experimental culture solutions, water samples from the culture solutions were taken in the beginning (placing of the nauplii in solutions) and at the end of the experiments (after 72 h) and analyzed by High Performance Liquid Chromatography (HPLC), according to the following in-house method, developed and described by the authors.

Water samples (200 μL) were diluted with 800 μL methanol in an Eppendorf tube, shaken manually, and filtered through Titan 2 HPLC nylon membrane filters (17 mm, 0.45 μm pore size) prior to injection into the HPLC system. Chromatographic analysis for determining BPA concentrations was performed with an HP 1100 liquid chromatograph (Hewlett-Packard GmbH, Waldbronn, Germany) equipped with a ternary-delivery system, a variable-wavelength UV detector, and an HP ChemStation LC 3D chromatography manager data acquisition and processing system (Hewlett-Packard GmbH, Waldbronn, Germany) with the ability to obtain UV spectra at a selected retention time of chromatograms. The analytical column was a Fortis UniverSil HS C_{18} column (150 × 3.0 mm I.D. with 5 μm particle size) with a guard column and was maintained at 40 °C. The mobile phase was methanol and water (with 5% methanol) 60/40 delivered at a flow of 1.0 mL min^{-1}, with an injection volume of 20 μL. The optimum detection was obtained at 230 nm. BPA concentrations were determined by the external standard technique by comparing the peak heights in the samples with those found in the calibration solutions (0.6 to 7.0 μg/mL).

2.5. Statistical Analysis

LC_{50} values for each replicate were calculated using the Probit method [18] and the LC_{50} for each exposure time was expressed as the mean of the respective values. Variations in values of LC_{50} and body measurements among exposure times were tested using one-way ANOVA, while individual group differences were assessed by Fisher's Least Significant Difference (LSD) procedure. The homogeneity of variances was tested prior to analyses with the Levene test. Additionally, the coefficient of variation (CV) was calculated, since the ratio between standard deviation (SD) and the overall mean of the body measurements. All statistical analyses were performed using STATGRAPHICS Centurion software package (The Plains, Virginia, USA).

3. Results

The recovery rate from chromatographic analysis for determining BPA concentrations was >90% so differences between nominal and detected concentrations, possibly caused by photodegradation, evaporation, or absorption, were considered negligible.

Results from all toxicity tests were acceptable, since control mortality did not exceed 10%. Mean values of LC_{50} for BPA were 45.51 ± 0.20 ppm for 24 h, 34.45 ± 0.39 ppm for 48 h, and 17.12 ± 0.90 ppm for 72 h. Differences were always statistically significant, both between and among exposure times ($F_{2, 28}$ = 5520.07, $p < 0.05$). For SDS, the respective mean values of LC_{50} were 8.49 ± 0.21 ppm for 24 h, 7.87 ± 0.26 ppm for 48 h, and 7.18 ± 0.23 ppm for 72 h. Again, differences were always statistically significant, both between and among exposure times ($F_{2, 28}$ = 68.79, $p < 0.05$). Our results indicated that BPA had lower toxicity than SDS. BPA had a wider range of LC_{50} values, ranging from 15 to 46 ppm, which is contrary to SDS. This was within 6–9 ppm. Both compounds had a strong correlation between mortality and concentration (Figure 2), with BPA having a better fit ($R^2 > 0.950$) than SDS ($R^2 < 0.950$).

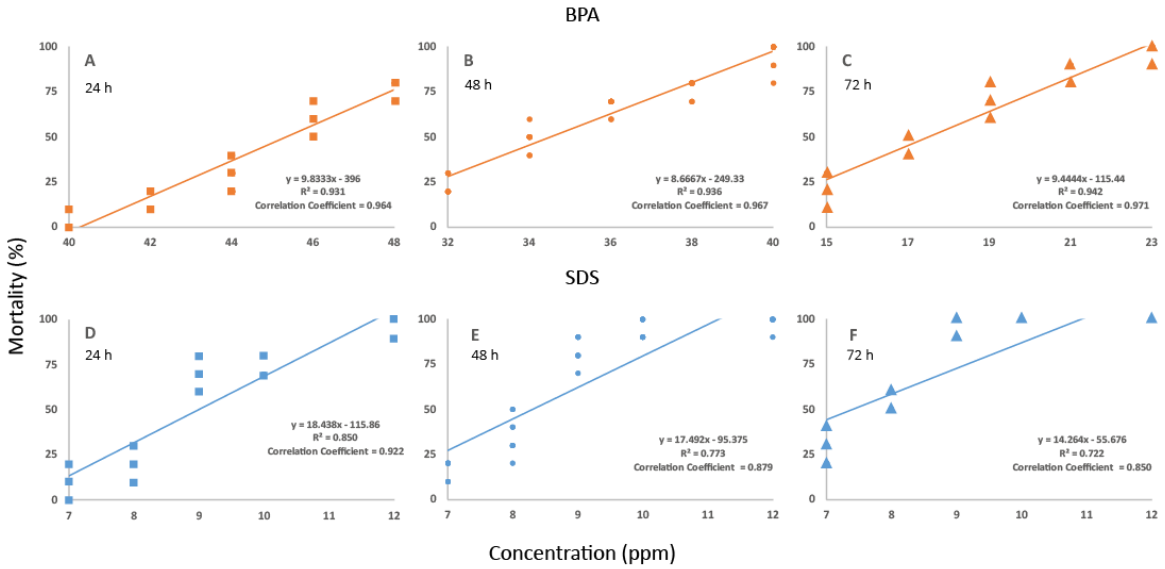

Figure 2. Concentration—Mortality curves and the estimated linear regression equations after exposure of *Artemia franciscana* nauplii to BPA for: (**A**) 24 h, (**B**) 48 h, (**C**) 72 h, and to SDS for: (**D**) 24 h, (**E**) 48 h, and (**F**) 72 h.

Results from the effect on the body growth of *A. franciscana* for BPA are presented in Table 1 and for SDS in Table 2. The CV values for all measurements were ≤ 10%, which indicates a good precision. Results indicated that body length of the nauplii decreased as concentrations of both toxicants increased. According to ANOVA results, body length values were statistically different among the different concentrations of both toxicants ($p < 0.05$). For BPA, F-values were $F_{8, 441} = 32.72$ for 24 h, $F_{8, 441} = 49.78$ for 48 h, and $F_{8, 441} = 44.84$ for 72 h of exposure. Post-hoc analysis revealed that mean values from the control showed statistically significant differences, at the 95.0% confidence level, with the values from concentrations ≥ 5 ppm, for all exposure times. For SDS, the F-values were $F_{8, 441} = 17.60$ for 24 h, $F_{8, 441} = 11.53$ for 48 h, and $F_{8, 441} = 8.69$ for 72 h of exposure. Post-hoc analysis revealed that mean values from the control showed statistically significant differences, at the 95.0% confidence level, with the values from concentrations ≥ 6 ppm for 24 h and 48 h of exposure, and with the values from all the concentrations ≥ 5 ppm for 72 h of exposure.

Apart from the inhibition of growth, no other malformations were recorded, except from the 72 h of exposure on BPA culture, where a significant abnormal growth of the central eye of the nauplii was detected (Figure 3). Results from the percentage of affected larvae displayed a strong linear increase (correlation coefficient > 0.90), which ranges from 2.67% at 10 ppm to 20.67% at 35 ppm concentration of BPA (Figure 4).

Table 1. Main summary statistics of body length measurements (mm) of *Artemia nauplii* exposed to different concentrations of BPA for 24, 48, and 72 h. * Significant differences. Means with different superscript letter(s) are significantly different.

Concentration (ppm)	24 h				48 h				72 h			
	Min	Max	Mean ± SD	CV (%)	Min	Max	Mean ± SD	CV (%)	Min	Max	Mean ± SD	CV (%)
0 (Control)	0.70	0.84	0.78 ± 0.04^{e}	4.82	0.76	0.89	0.83 ± 0.06^{e}	4.29	0.82	1.09	0.93 ± 0.06^{d}	6.03
3	0.70	0.82	0.76 ± 0.03^{e}	4.21	0.78	0.85	0.82 ± 0.06^{e}	3.20	0.86	1.01	0.92 ± 0.06^{d}	6.06
5	0.62	0.82	0.75 ± 0.05^{d}	7.28	0.68	0.90	0.80 ± 0.05^{d}	6.29	0.76	0.97	0.89 ± 0.05^{c}	6.15
8	0.68	0.77	0.72 ± 0.03^{c}	4.73	0.73	0.80	0.77 ± 0.05^{c}	3.18	0.79	0.94	0.87 ± 0.05^{c}	5.24
10	0.66	0.77	0.71 ± 0.04^{bc}	4.96	0.70	0.78	0.74 ± 0.04^{b}	3.02	0.75	0.91	0.84 ± 0.04^{b}	4.85
15	0.61	0.77	0.70 ± 0.05^{b}	6.52	0.66	0.87	0.72 ± 0.05^{ab}	7.21	0.71	0.89	0.83 ± 0.05^{b}	6.09
20	0.65	0.76	0.70 ± 0.04^{ab}	5.51	0.64	0.88	0.72 ± 0.05^{a}	7.51	0.69	0.87	0.80 ± 0.05^{a}	6.07
25	0.61	0.79	0.68 ± 0.05^{a}	7.74	0.63	0.85	0.71 ± 0.07^{a}	8.45	0.70	0.93	0.80 ± 0.07^{a}	8.22
35	0.61	0.79	0.68 ± 0.05^{a}	6.58	0.60	0.85	0.71 ± 0.06^{a}	10.00	0.70	0.93	0.80 ± 0.06^{a}	7.50
F-Ratio			32.72				49.78				44.84	
p value			<0.001 *				<0.001 *				<0.001 *	

Table 2. Main summary statistics of body length measurements (mm) of *Artemia nauplii* exposed to different concentrations of SDS for 24, 48, and 72 h. * Significant differences. Means with different superscript letter(s) are significantly different.

Concentration (ppm)	24 h				48 h				72 h			
	Min	Max	Mean ± SD	CV (%)	Min	Max	Mean ± SD	CV (%)	Min	Max	Mean ± SD	CV (%)
0 (Control)	0.62	0.81	0.71 ± 0.04^{e}	6.09	0.71	0.99	0.84 ± 0.08^{d}	8.99	0.83	1.00	0.93 ± 0.04^{c}	4.35
4	0.62	0.79	0.71 ± 0.04^{e}	5.52	0.72	0.98	0.83 ± 0.08^{d}	9.19	0.83	1.00	0.92 ± 0.04^{bc}	4.50
5	0.62	0.76	0.7 ± 0.03^{e}	4.19	0.72	0.96	0.83 ± 0.07^{d}	8.59	0.79	1.00	0.90 ± 0.06^{b}	6.35
6	0.60	0.79	0.68 ± 0.04^{d}	5.52	0.69	0.92	0.80 ± 0.08^{c}	9.51	0.74	1.01	0.87 ± 0.08^{a}	8.76
7	0.60	0.78	0.68 ± 0.04^{cd}	5.58	0.67	0.91	0.79 ± 0.06^{c}	7.46	0.60	1.01	0.86 ± 0.07^{a}	8.67
8	0.59	0.77	0.67 ± 0.04^{bcd}	5.60	0.63	0.90	0.77 ± 0.05^{abc}	7.12	0.65	1.04	0.87 ± 0.07^{a}	7.70
9	0.58	0.75	0.66 ± 0.04^{ab}	5.65	0.66	1.02	0.79 ± 0.06^{bc}	7.12	0.71	1.01	0.86 ± 0.07^{a}	7.87
10	0.58	0.72	0.67 ± 0.03^{abc}	4.47	0.62	0.93	0.76 ± 0.06^{ab}	8.11	0.73	1.00	0.87 ± 0.06^{a}	7.32
12	0.58	0.73	0.65 ± 0.03^{a}	5.18	0.63	0.88	0.76 ± 0.06^{a}	8.49	0.70	0.99	0.87 ± 0.07a	8.45
F-Ratio			17.60				11.53				8.69	
p value			<0.001 *				<0.001 *				<0.001 *	

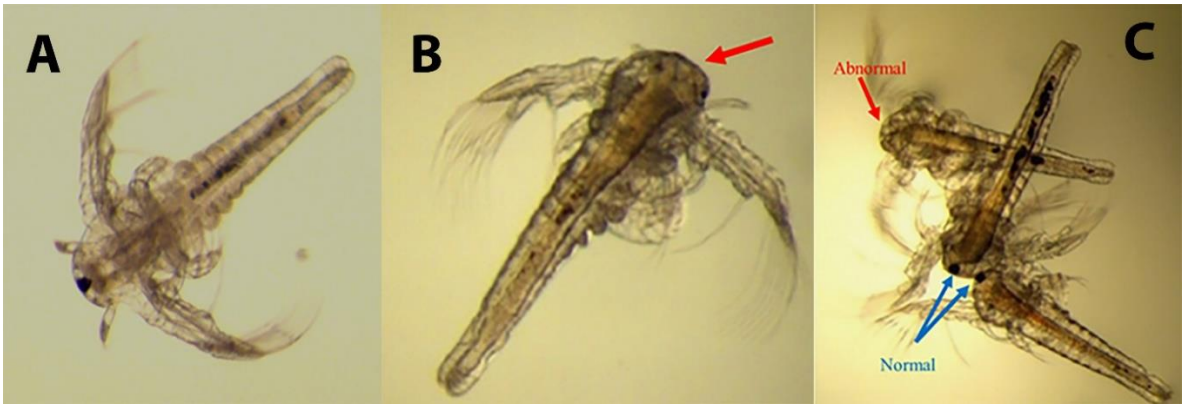

Figure 3. Digital photos from *Artemia franciscana* nauplii (**A**). Unaffected nauplius (**B**). Nauplius with obvious structural abnormality on the naupliar eye after 72 h of exposure to BPA (**C**). Comparative image of an *A. franciscana* individual after 72 h of exposure in BPA and unaffected individuals.

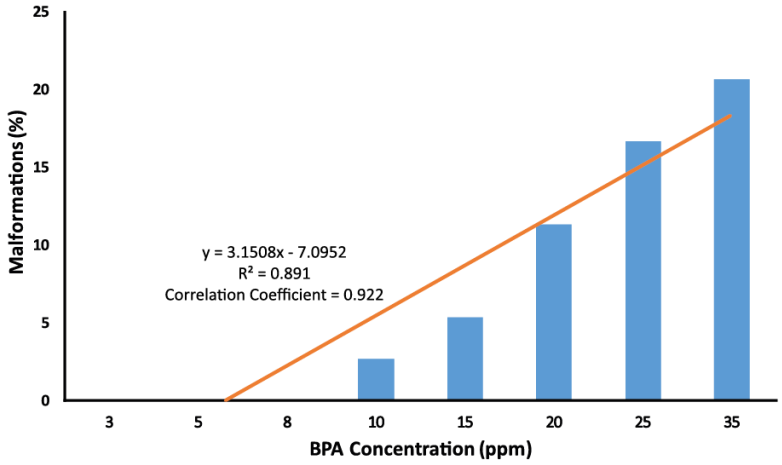

Figure 4. Percentage of *Artemia franciscana* nauplii individuals with structural malformations on the naupliar eye after 72 h of exposure to different concentrations of BPA.

4. Discussion

Despite some criticism [23], *Artemia* spp. is widely used as a test organism in a large number of ecotoxicology studies [7,10,14]. Short-term toxicity tests are the most common among studies and, usually, they include various endpoints such as survival, hatching, and behavioral patterns [24]. The majority of cyst-based toxicity assays on *Artemia* refer to a well-accepted endpoint as a criterion, which is known as the mortality of recently hatched nauplii (instar II-III stage) [7]. To promote alternative endpoints for *Artemia*, mortality and inhibition of growth of freshly hatched nauplii (Instar I-II) have been evaluated by using two well-known reference toxicants.

Results from mortality tests in the present study are consistent with other similar studies. For example, acute toxicity tests of SDS on *A. salina* reported that the median lethal dose (LD_{50}) values were 6.92 ppm for 24 h and 7.30 ppm for 48 h [25]. LC_{50} values for *Artemia* strains of San Francisco were 13.84 ppm for 24 h of exposure [26], LC_{50} values of *Artemia parthenogenetica* nauplii after 48 h of exposure were 12.20 ppm [18], and, more recently, *A. franciscana* LC_{50} values for 24 h and 48 h of exposure were 19.41 ppm and 15.60 ppm, respectively [9]. It is worth mentioning that all these studies used *Artemia* nauplii from the instar II-III stages, whereas the present study used Instar I-II as the test organism. With regard to the toxicity of BPA, the LC_{50} values from our study agree with the results from a similar study, which reported LC_{50} values of 44.8 ppm for 24 h and 34.7 ppm for 48 h, using *Artemia* nauplii instar II-III [13]. Even though Instar II-III stages are considered to be more sensitive [7], our results showed that this might not be the case, since LC_{50} values were lower compared to most of

the other studies. Furthermore, higher rates of mortality were observed after the first 24 h of exposure, as demonstrated by the higher slope values from the estimated linear regression equations, for both toxicants. Therefore, Instar II could be considered as the most vulnerable developmental stage, since nauplii have already undergone a molting and have started to filter nanoparticles from the surrounding environment by the second antennae and to ingest them into their functional digestive tract, which is more susceptible to potential intoxication effects.

Development of the nauplii was clearly influenced by the duration of exposure and growth inhibition was detected for both toxicants. Length of *Artemia* nauplii was decreased as the concentration of both tested compounds increased. Similar effects were detected in other studies, such as the growth inhibition observed after exposure of *A. franciscana* Instar II-III nauplii to different BPA concentrations [13] or antifouling paints [17]. Inhibition of *Artemia* length is considered as teratogenesis and can be used as an indicator of toxicity [11].

Another interesting finding in our study was the abnormal growth of the central eye of several *Artemia* nauplii after 72 h of exposure to BPA. Such changes in eye formation have been initially observed during a developmental study of *A. franciscana* during a space flight, where some animals appeared to have affected central eye growth, but observations were not considered as statistically significant [27]. Recently, a study involving exposure of *A. salina* with nanoparticles of titanium dioxide (TiO_2) and silver with titanium oxide ($AgTiO_2$) for 24 h recorded changes in the shape of the eye including shrinking and discoloration [28]. Furthermore, eye malformations in crustaceans were observed in *Daphnia magna* after exposure in demethylase inhibiting fungicides (DMIs) [29]. DMIs are similar to BPA and are suspected to disrupt endocrine-mediated processes inhibiting ecdysteroid biosynthesis, which is a pathway that has been shown to affect eye development in arthropods [30]. Lastly, BPA has also been implicated in eye malformations in vertebrate embryonic systems such as *Xenopus* spp. disrupting the Pax-6 and ESR-1 genes that are responsible for the development of eyes and the central nervous system [31].

Our findings confirmed the hypothesis of growth inhibition on *Artemia* nauplii from exposure to BPA and SDS. Although our results do not relate, directly, to either human or fisheries health, they reinforce the notion that *Artemia* can be used as a simple and accurate tool in bioassays for water quality testing. Therefore, indirectly, such tests could help detect pollutants in water bodies that would potentially affect human or fisheries health. Future research should focus on the effects of other pollutants on *Artemia* spp. growth, to further reinforce the value of somatic growth as an endpoint in toxicity studies and, also, to identify the effects of those pollutants in the mechanisms related to growth and development of *Artemia* nauplii, at the molecular level. Furthermore, research focus should be shifted to the study of the potential transmission of toxicity effects from exposed parents to their offspring, which is a rather new and controversial topic [32,33] that the authors are already investigating.

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