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Toxicity effects of bisphenol A to the nauplii of the brine shrimp *Artemia franciscana*

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Toxicity responses of *Artemia franciscana* to the endocrine disrupter bisphenol A (BPA) were investigated. Exposure of *Artemia* nauplii (instar II-III) to BPA for 24 hrs and 48 hrs demonstrated LC_{50} values of 44.8 mg l^{-1} and 34.7 mg l^{-1}, respectively. The length of nauplii was measured and compared with that of untreated animals. BPA exposed nauplii were significantly shorter than untreated individuals (24 hrs: 0.97 mm, 48 hrs: 1 mm) at a concentration range between 20 and 50 mg l^{-1} (24 hrs: 0.9-0.7 mm, 48 hrs: 0.92-0.71 mm). Furthermore, the length of nauplii decreased as the dose of BPA increased for both 24 hrs and 48 hrs exposure periods. The results indicate that *A. franciscana* does not consist a highly sensitive test animal for the acute toxicity bioassays with BPA in comparison to other aquatic organisms. However, it becomes obvious that an inhibitory effect on growth of *Artemia* nauplii can be estimated within a short exposure period (24 hrs), even at doses lower than the median lethal concentration. The latter finding points out that *A. franciscana* may be an ideal model organism for further research on the physiological processes related to the inhibitory effect of BPA on the growth of crustaceans.

Key words: Bisphenol A, *Artemia* nauplii, lethal concentration, length, inhibitory effect.

INTRODUCTION

Bisphenol A (BPA, 2, 2-bis (4-hydroxyphenyl) propane; CAS# 80-05-07) is extensively used in the production of polycarbonate plastics and the majority of epoxy resins, as well as an additive to other plastics and as a component of fire retardants (Markham *et al.*, 1998; Staples *et al*., 1998). The annual production of BPA in USA reached a volume of 2.4 billion pounds in 2007 (Environmental Protection Agency, USA; http://www.epa.gov/oppt/existingchemicals/pubs/actionplans/bpa.html). Safety of BPA has been a controversial issue, since it has been found to be an endocrine disruptor due to its ability to interfere with endocrine activity and is considered to be a reproductive, developmental and systemic toxicant in humans, experimental animals and wildlife (Mountfort *et al.*, 1997; Yamamoto & Yasuhara, 1999; Hirano *et al*., 2004; Mihaich *et al*., 2009).

To date there are reports describing the detection of BPA in soil, while it is found widely dispersed in the atmosphere (Fent *et al*., 2003; Fu & Kawamura, 2010). Furthermore, considerable amount of BPA toxicity testing has been focused on aquatic organisms, since the discharge from production, processing and sewage treatment plants effluxes in aquatic environments (Cousins *et al*., 2002). The majority of testing

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has been undertaken on studies involving fresh water fish (Caunter, 1998; Caunter *et al*., 2000; Yokota *et al*., 2000; Sohoni *et al*., 2001) and invertebrates (Hill *et al.*, 2002; Hirano *et al*., 2004). In response to the EU Technical Guidance document, Mihaich *et al.* (2009) proposed a number of taxonomically diverse species for BPA toxicity assessment in fresh water environments.

However, the effects of BPA to marine organisms and the health of human consumer might also be under concern, since accumulation has been demonstrated in edible marine fish and seafood (Basheer *et al*., 2004; Mita *et al*., 2011). Previously, the effects of BPA on marine life have been demonstrated mainly on molluscs, but also on crustaceans and vertebrates (Hirano *et al*., 2004; Ozlem & Hatice, 2008; Zhou *et al*., 2011). Crustaceans and molluscs appear to be more sensitive than fish to BPA and moreover the formers show developmental disturbances (Oehlmann *et al*., 2009). To date, the effects of BPA on the larval development of marine planktonic crustaceans have been limitedly studied, mainly on that of the copepod *Acartia tonsa* (Andersen *et al.*, 2001; Oehlmann *et al.*, 2009). However, *A. tonsa* shows a restricted distribution to estuarine and coastal waters and it is sensitive against increases in salinity (Cervetto *et al*., 1999).

The aim of this study was to estimate the effects of BPA on the survival and growth of early larval stages (instar II-III) of the crustacean *Artemia*, a marine non-target organism that has been very well adapted to the laboratory practice. Brine shrimp larvae are commonly used for cytotoxicity assays in pharmacology (Pelka *et al*., 2000), for testing marine natural products (Carballo *et al*., 2002) or antifouling paints (Okamura *et al*., 2000; Hadjispyrou *et al*., 2001; Katranitsas *et al*., 2003; Castritsi-Catharios *et al*., 2007), for assessing potential toxicity of epiphytic species (Aligizaki *et al*., 2009) as well as live feed in aquaculture (Sorgeloos *et al*., 1998). We also evaluate the usefulness of BPA toxicity testing with a marine organism that would require ease of application, due to its ability to create a dormant state during its reproductive part of its life cycle, precision, ability to repeat toxicity tests with little variability, reduced scale of organisms, reduced test volumes, minimal amount of produced waste and space needed to perform testing protocols (Blaise, 1998).

There are two additional significant advantages of the organism and its developmental stages selected for our experiments. The first is that *Artemia* nauplii (instar I) are not fed externally and nauplii 24 hrs after hatching (instar II-III) may remain unfed for a further time interval of 48 hrs without obvious problems, while may exhibit increased mortality after 72 hrs of starvation (Castritsi-Catharios *et al*., 1987). Thus, the effect of the nutritional status of the specimens (nauplii instar II-III) prior or during the static toxicity bioassays (up to 48 hrs) could be avoided, which in turn affects the produced results. Bengston *et al.* (1984) reported that one of the most important variables in the performance of any biological experiment is the nutrition of the test animals used. The second advantage is the short period within which brine shrimp grows by performing several molts; e.g. the adult stage is reached about three weeks after hatching by the completion of the 17th molt (Drewes C; http://www.eeob.iastate.edu/faculty/DrewesC/htdocs/ARTEMIA.PDF). At the high water temperatures that are applied during cyst incubation, freshly hatched *Artemia* nauplii may develop into the second larval stage within 6 to 8 hrs (Sorgeloos *et al*., 1998). Also, two-day old nauplii grown at 28-30°C may reach the fourth larval stage (Makridis & Vadstein, 1999). In addition, the developmental stages of *Artemia* are well distinguished by several morphological characters (Schrehardt, 1987; Criel & MacRae, 2002).

A serious argument is the sensitivity of *Artemia* and its usefulness as a reliable test animal. It is known that its sensitivity, like most aquatic organisms, depends on the developmental stage and its origin (Castritsi-Catharios *et al.*, 1982, 1987). Castritsi-Catharios *et al.* (1980) reported that dehydrated cysts are not sensitive against specific pollutants and probably *Artemia* is not a reliable test organism, despite all its other advantages. On the contrary, Brix *et al*. (2006) suggested that the hatching end point for *A. franciscana* seems the most sensitive up to date for testing the toxicity of Cd and Zn in saline environment.

Most frequently, cyst-based toxicity assays involving *Artemia* refer to a well-accepted end point as criterion: mortality of the recently (instar II-III) hatched larvae (Nunes *et al.*, 2006). These instars have, in several papers, been shown to be the most sensitive stage (Sorgeloos *et al.*, 1978; Vanhaecke & Persoone, 1984; Sánchez-Fortún *et al*., 1996). Furthermore, Castritsi-Catharios (1987) claimed that *A. franciscana* (Carolina, USA) freshly-hatched nauplii were more sensitive compared to those of a parthenogenetic *Artemia* population (Messolonghi, Greece) to a dispersant and its mixture with gas-oil. Due to both the geographical origin and type (bisexual or parthenogenetic) of *Arte-*

mia populations, acute toxicity tests may lead to wrong results because of differences in the sensitivity of the cysts (Browne, 1980; Varó *et al.*, 1998). Nunes *et al.* (2006) concluded that the adaptability of *Artemia* genus to distinct environmental conditions might turn *Artemia*, in the future, into a crucial test organism for ecotoxicology testing, particularly for saline environments and provided suggestions to overcome problems related to toxicity assessment. For the aforementioned reasons, in this study the instars II-III of the bisexual *A. franciscana* were selected to be exposed to BPA.

MATERIALS AND METHODS

Acute toxicity tests were performed by slightly modifying the standard method developed at the *Artemia* Reference Center (ARC-Test) in the Laboratory for Mariculture at the State University of Ghent Belgium (Vanhaecke & Persoone, 1984). As test organism, a well-defined *Artemia* strain was used (1710, Great Salt Lake, Utah, USA). Cysts were hatched under standard conditions (temperature chamber: 25 ± 0.5°C, continuous illumination). Sea water was prepared by using artificial salt mixture of Instant Ocean®, dissolved in distilled water, filtered through a 1 μm filter and aerated (salinity 35 ± 1 g l^{-1}, pH 8.0 ± 0.5 and oxygen content above 90% saturation). Nauplii at instar II-III stages (48 hrs after the start of the incubation) were used in our experiments. The developmental stages were confirmed through several morphological characteristics of samples, like gnathobasic setae (Abatzopoulos, 1989). The age homogeneity of the population was checked in each test under a binocular stereoscope.

BPA solutions of different concentrations were made according to the method described by Fan *et al.* (2007). Then, 4 ml of each solution were transferred to a well on a multi-well testing plate (Castritsi-Catharios *et al*., 2007) (instead of Petri dishes previously used in ARC-Test). Specifically, in each plate (twelve wells), the controls were placed in the three wells of the first column (each well contained 4 ml pure artificial sea water), while in each of the remaining nine wells 4 ml of the different BPA solutions (one concentration per column of three wells) were added. Twelve instar II-III larvae were transferred with a Pasteur pipette (minimizing the water volume) in each well and exposed to PBA for 24 hrs or 48 hrs. The plate was covered and larvae remained unfed during the bioassays. Static acute toxicity tests were conducted in a constant temperature chamber (25°C) under continuous illumination. At the end, biometry was performed under a binocular microscope equipped with a micrometric scale. The length of the live larvae was measured. Larvae were considered dead if they do not exhibit any internal or external movement in about 10 s of observation.

A series of preliminary tests were performed in a broad scale of BPA nominal concentrations (1, 5, 10, 15, 20, 30, 40, 50, 100, 150 and 200 mg l^{-1}) for determining those that cause average mortality higher than 20% and lower than 80% (data not shown) after 24 or 48 hrs of exposure. A definitive series of tests was followed within the critical range of the organism depending on the time of exposure, which was taken under consideration for the calculation of the median lethal concentration after 24 hrs or 48 hrs (LC_{50}: concentration of toxicant that kills the 50% of the test animals after exposure for a certain time period).

For the estimation of the LC_{50}, 10-15 replicates were used at each tested concentration of BPA. Regression analysis was applied on the data of mortality of *Artemia* nauplii *versus* the dose of BPA after exposure for 24 hrs or 48 hrs, in order to estimate the respective LC_{50} values according to Bliss (1938). Regression analysis was also performed on the biometry data *versus* the dose of BPA for both time periods of exposure in the definitive tests. Normal distribution was assessed before regression analysis (Kolmogorov test). The significance ($p < 0.05$) of the differences between the regression coefficients, as well as between the intercepts, was tested (ANOVA) for the length obtained after 24 hrs or 48 hrs exposure of larvae to different concentrations of BPA. The morphometric data recorded at 0, 20, 30, 40 and 50 mg l^{-1} of BPA in the preliminary tests were also analyzed using the non-parametric tests of Mann-Whitney test for two sample comparisons and Kruskal-Wallis test for multiple sample comparisons. All analyses were performed using Statgraphics Plus version 4 (Manugistics Group, Inc).

RESULTS AND DISCUSSION

Bisphenol A toxicity has been studied extensively in literature; however, this is the first report on the effect on *Artemia* larvae. The LC_{50} values and their 95% confidence limits (LCL-UCL), calculated by the respective linear regression equations of BPA doses on the mortality of *Artemia* nauplii after exposure for 24 hrs or 48 hrs (Fig. 1), were 44.8 (44.6-45) mg l^{-1}

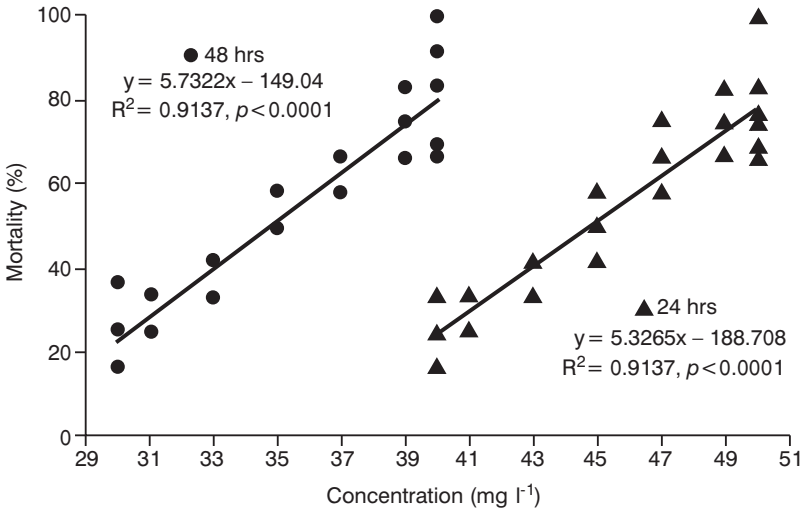

FIG. 1. Dose-mortality curves and the estimated linear regression equations of BPA doses (x) on (%) mortality (y) of *Artemia* nauplii after exposure for 24 hrs or 48 hrs.

and 34.7 (34.5-34.9) mg l^{-1}, respectively. At 40 mg l^{-1} of BPA the mortality (%) after 48 hrs of exposure was much higher than that after 24 hrs, confirming that the elongation of the exposure time enhances the toxicity effects. LC_{50}, EC_{50} and IC_{50} values of BPA reported previously for other aquatic organisms are much lower compared to those estimated in this study for *Artemia* nauplii. They vary between 4.6 to 17.93 mg l^{-1} for fish and 0.3 to 10 mg l^{-1} for invertebrate species (Staples *et al.,* 1998; Ozlem & Hatice, 2008; Mihaich *et al.*, 2009). This difference in LC_{50} value for *Artemia* could be possibly attributed to adaptive mechanisms and higher resilience in environmental pressures.

Biometric parameters of *Artemia* cysts and nauplii such as length, volume and weight are characteristic for each particular strain and are considered to be tools for strain characterisation (Vanhaecke & Sorgeloos, 1980). Furthermore, Hirano *et al.* (2004) in their study of acute toxicity of endocrine disrupters on the aquatic crustaceans *Americamysis bahia* and *Daphnia magna* pointed out that additional endpoints such as growth, reproduction and molting should be considered in the future. In this study, the length of *Artemia* nauplii decreased as the concentration of BPA increased from 30 to 40 mg l^{-1} or 40 to 50 mg l^{-1} after exposure for 48 hrs or 24 hrs, respectively, in comparison to controls (Fig. 2). In both cases, the estimated linear regression equations were highly significant ($p < 0.0001$). In addition, comparison of these two regression lines showed significant differences between the intercepts ($p < 0.0001$) and the slopes ($p < 0.01$). This indicates that the growth of nauplii was more severely affected after 48 hrs than 24 hrs exposure to BPA, particularly at doses higher than the LC_{50} at 48 hrs.

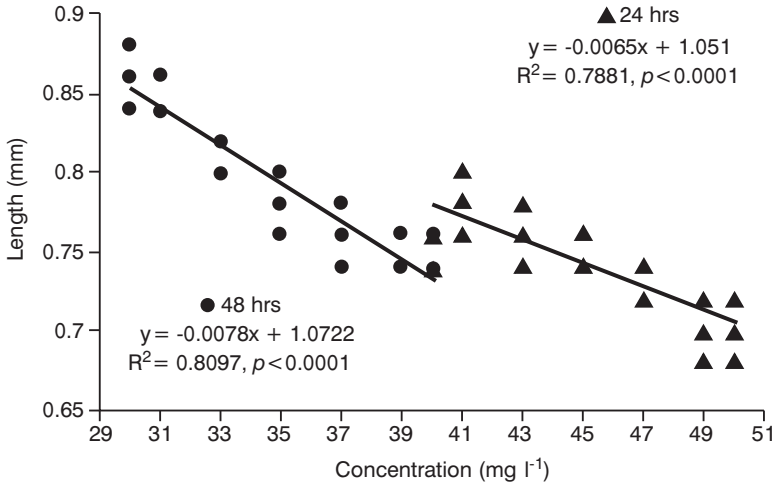

FIG. 2. Dose-length curves and the estimated linear regression equations of BPA doses (x) on the length (y) of *Artemia* nauplii after exposure for 24 hrs or 48 hrs.

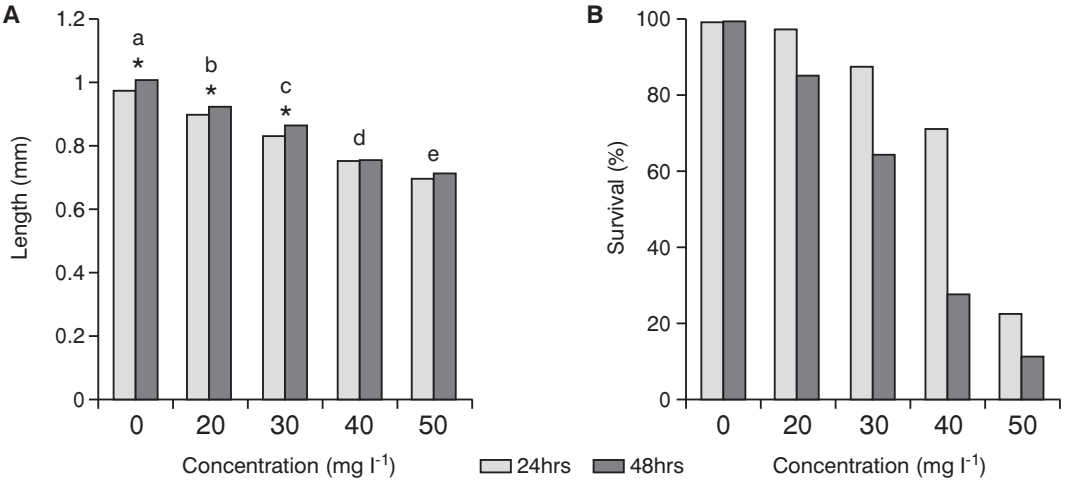

FIG. 3. (A) Mean values of the length measured on *Artemia* nauplii exposed to different concentrations of BPA for 24 hrs or 48 hrs. Different letters indicate significant differences ($p < 0.05$) among the concentrations for both exposure periods and asterisks between 24 hrs and 48 hrs exposure at each concentration. (B) Average survival (%) of *Artemia* nauplii exposed to different concentrations of BPA for 24 hrs or 48 hrs.

At 20 mg l^{-1} of BPA the mean length of *Artemia* nauplii exposed for 24 hrs (0.9 mm) or 48 hrs (0.92 mm) was significantly lower than that of the respective controls (24 hrs: 0.97 mm, 48 hrs: 1 mm) (Fig. 3A). Further increase of the BPA dose up to 50 mg l^{-1} provoked significant reductions in the length every 10 mg l^{-1} for both exposure periods. Moreover, the animals were significantly shorter in length at concentrations 20 and 30 mg l^{-1} of BPA after 24 hrs exposure than at the same concentrations after 48 hrs exposure. However, at concentrations of 40 and 50 mg l^{-1} of BPA, no significant effects were found between the two time periods of exposure. It seems that these BPA concentrations greatly inhibited the growth of *Artemia* nauplii, since their size remained at almost similar levels from 24 hrs to 48 hrs of exposure. We must point out that the controls exhibited one molt after 24 hrs exposure and, at least, two molts after 48 hrs exposure. Specifically, the length of the controls in the 24 hrs toxicity bioassays was similar to that of two-day old fed nauplii of *A. franciscana* (Makridis & Vadstein, 1999). On the contrary, at 50 mg l^{-1} of BPA the length of the nauplii was found at levels reported for instars II-III of several populations of *Artemia* (Moraiti-Ioannidou *et al*., 2007).

Although the length of *Artemia* nauplii was reduced at 20 mg l^{-1} of BPA, the average survival (%) was found at relatively high levels after 24 hrs (98.2%) or 48 hrs (85.2%), as well as at 30 mg l^{-1} of BPA after 24 hrs (87.5%) of exposure (Fig. 3B). Thus, it becomes obvious that an inhibitory effect on growth of *Artemia* larvae can be estimated within a short time period of exposure (24 hrs), even at doses lower than the median lethal concentration.

Berges *et al.* (1990) tested several key enzymes in *Artemia* larval development and demonstrated that size of larvae is strongly associated with the activity of nucleoside diphosphate kinase. Although the specific effect of BPA on nucleoside phosphate kinase is unresolved, it has been shown that other polyphenols exhibit a strong inhibitory effect (Malmquist *et al*., 2001). Another possibility for BPA action on *Artemia* may be related with cascade mechanisms of ecdysis and growth of the organism. In arthropods, including crustaceans, ecdysosteroids, such as 20-hydroxyecdysone, participate as endocrine-signaling molecules and function in the control of molting, reproduction and embryogenesis (LaFont, 2000; LaFont & Mathieu, 2007). BPA has been demonstrated as a weak ecdysteroid antagonist in insects (Dinan *et al*., 2001) and in daphnids (Mu *et al*., 2005). Interestingly, exposure of *Artemia* in municipal effluents with ecdysosteroid characteristics, as well as in 20-hydroxyecdysone, resulted in increasing acetylcholinesterase activity and changes in the shell protein composition, interactions that are associated with embryogenetic events (Gagné & Blaise, 2004).

In this study we investigated the responses of *Artemia* nauplii to BPA activity. Overall, this study suggests that *Artemia* is resilient to BPA in comparison to other aquatic organisms. However, it demonstrates detrimental effects of BPA on the nauplii size, which

may be associated with alterations in the activity of specific enzymes and shell protein composition involved in the development and growth of *Artemia*. Further research is required on this issue, as *Artemia* seems to be a suitable organism for testing sublethal effects of BPA, particularly on the mechanisms of ecdysis in crustaceans.

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