Bisphenol A migration from plastic materials: direct insight of ecotoxicity in *Daphnia magna*

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Abstract Bisphenol A (BPA) is an endocrine disrupting chemical (EDC) whose migration from food packaging is recognized worldwide. However, the real overall food contamination and related consequences are yet largely unknown. Among humans, children's exposure to BPA has been emphasized because of the immaturity of their biological systems. The main aim of this study was to assess the reproductive impact of BPA leached from commercially available plastic containers used or related to child nutrition, performing ecotoxicological tests using the biomonitoring species *Daphnia magna*. Acute and chronic tests, as well as single and multigenerational tests were done. Migration of BPA from several baby bottles and other plastic containers evaluated by GC-MS indicated that a broader range of foodstuff may be contaminated when packed in plastics. Ecotoxicological test results performed using defined concentrations of BPA were in agreement with literature, although a precocious maturity of daphnids was detected at 3.0 mg/L. Curiously, an increased reproductive output (neonates per female) was observed when daphnids were bred in the polycarbonate (PC) containers (145.1±4.3 % to 264.7±3.8 %), both in single as in multigenerational tests, in comparison with the negative control group (100.3±1.6 %). A strong correlated dose-dependent ecotoxicological effect was observed, providing evidence that BPA leached from plastic food packaging materials act as functional estrogen in vivo at very low concentrations. In contrast, neonate production by daphnids cultured in polypropylene and non-PC bottles was slightly but not significantly enhanced (92.5±2.0 % to 118.8±1.8 %). Multigenerational tests also revealed magnification of the adverse effects, not only on fecundity but also on mortality, which represents a worrying trend for organisms that are chronically exposed to xenoestrogens for many generations. Two plausible explanations for the observed results could be given: a non-monotonic dose–response relationship or a mixture toxicity effect.

Keywords Endocrine disruptors · Bisphenol A · Food packaging · Low-dose effects · Mixture effects · Daphnids · Ecotoxicological tests

Introduction

Plastic materials are considered extremely convenient for food storage, preparation, and consumption.

However, potential harmful substances can be leached from plastics and released into water and food matrices, especially at high temperatures (pasteurization, sterilization, or microwave heating; Erler and Novak 2010; Maia et al. 2009). This issue was focused recently by the European Union (EU; SEC 2011) and other international organizations (EFSA 2012; USFDA 2012) that have shown concern about the real overall food contamination and cumulative effects of such substances. Some of the leached compounds are recognized or suspected to be endocrine disrupting compounds (EDCs), like bisphenol A (BPA). BPA is a chemical component of epoxy resins used in protective coatings and linings for food and beverage cans and vats, and is also a component of polycarbonate (PC), a plastic used to produce food containers, beverage bottles, infant feeding (baby) bottles and tableware (plates and mugs; EFSA 2012; Nam et al. 2010; USFDA 2012; Viñas et al. 2010).

BPA has been associated with increased incidence of cardiovascular disease, diabetes, reproductive cancers, fertility problems and other endocrine-related endpoints, even at a very low-level exposure, which remains a major challenge for scientists and regulators (Canada Environment 2009; Cunha and Fernandes 2010; De Coensel et al. 2009; EC 2010; Ehrlich et al. 2011; Erler and Novak 2010; Kortenkamp et al. 2012; Meeker et al. 2010; Wagner and Oehlmann 2009; USEPA 2010). In January 2011, the European Commission adopted the Directive 2011/8/EU as regards the restriction of use of BPA in plastic infant feeding bottles (EC 2011). Nevertheless, there are still hundreds of products on the market commercialized without any surveillance. In February 2012, following further consideration of new scientific studies, the European Food Safety Authority (EFSA) decided to undertake a full re-evaluation of the human risks associated with exposure to BPA through food contact materials, focusing in particular on possible low-dose effects and on the exposure of vulnerable groups, particularly at sensitive life cycle stages, as infants and children, that appear to be exposed to BPA primarily through food packaging materials and feeding products (EFSA 2012).

As a consequence of severe restrictions on substances identified as endocrine disruptors imposed by some pieces of legislation (as for example: Regulation (EC) No. 1907/2006; Regulation (EC) No. 1107/2009 and Regulation (EU) No. 528/2012), significant efforts have also been made at international level to accurately identify and assess endocrine disruptors' hazards. The Conceptual Framework (CF) from the Organisation for Economic Co-operation and Development (OECD) for testing and assessment of EDCs was recently reviewed in the light of the scientific progress obtained in this area (OECD 2011). Presently, it comprises of an Integrated Testing Strategy with five levels of organization. Level 5 consists of whole-organism assays that provide more comprehensive data on adverse effects on relevant endpoints over more extensive parts of the life cycle of an organism. Invertebrate life cycle tests were added, including the *Daphnia* 21-day reproduction test or TG211, that was recently reviewed (OECD 2012). *Daphnia magna* is used since 1980 as a biomonitoring species, being an approved test model due to their sensitivity towards pollutants (ASTM 2004; OECD 1984, 2004, 2012; USEPA 2002).

Despite the actual controversy about how to interpret results of the *Daphnia* tests for identification, risk assessment, and testing strategy of EDCs (Brennan et al. 2006; Oetken et al. 2004), *D. magna* seems to be a valuable model for evaluating endocrine disruptors' effects. In the Final Report of the State of the Art Assessment of Endocrine Disrupters (Kortenkamp et al. 2012), it is stated that assays utilizing invertebrates offer some advantages over vertebrate models, namely that there are fewer ethical considerations when using invertebrates, doses are easier to deliver precisely in aquatic animals, and their small size and inexpensive cultural requirements allow larger datasets to be collected. Although few have been explored so far, invertebrate models have much to offer both as a means for investigating EDC effects relevant to vertebrates and as ecotoxicological models for screening chemicals for potential endocrine disrupting properties.

Two major goals were established for this study. The first one was to test several plastic containers used to feed children, such as baby bottles, an infant feeding cup, and some disposable and microwave-reheatable containers, since it is a household practice to store and freeze homemade baby food, as soups and purees, in little plastic boxes. Migration experiments were done under defined time and temperature conditions, as it was intended to simulate routine applications (boiling, washing, and reuse), using a gas chromatography–mass spectrometry (GC-MS) method that was developed and validated according to international conventions to provide an accurate quantification of very low levels of BPA (Mansilha et al. 2010, 2012). The other goal of this study was to assess the impact of BPA on the crustacean *D. magna* performing preliminary acute and chronic tests according to OECD (2004, 2012), and then investigating the impact of direct exposure on reproductive endpoints of daphnids bred in the plastic containers. These last experiments were similar to those of Martin Wagner and Jörg Oehlmann that provided the first evidence that substances leaching from plastic food packaging materials act as functional estrogens in vivo (Wagner and Oehlmann 2009). Transgenerational chronic effects were also observed. Multigenerational experimental designs were employed to account for continuous exposure to BPA and, eventually, to other plastic reaching materials, in order to assess the long-term chronic effects over several generations.

Material and methods

Chemicals and reagents

Bisphenol A, 2,2′-bis(4-hydroxyphenyl)propane CAS Number 80-05-7, ≥99 %, deuterated-BPA CAS Number 96210-87-6 (BPA-d_{16}), and 2,2,2-trifluoro-N-methyl-N-(trimethylsilyl)acetamide CAS Number 24589-78-4, >98 % (MSTFA) were supplied by Sigma-Aldrich (Steinheim, Germany). Acetic acid (glacial) 100 % was supplied by Merck (Darmstadt, Germany). Methanol and ethyl acetate were of organic trace analysis grade (SupraSolv) and were also supplied by Merck. Strata SDB-L (200 mg, 3 mL) cartridges were from Phenomenex (Torrance, CA, USA). Ultra-pure water (0.054 μS/cm) was obtained by using a Milli-Q system from Millipore (Milford, MA, USA).

ASTM synthetic hard water (ASTM 2008) was used as culture medium for *D. magna*. The rearing procedures followed the recommendations of standard protocols (OECD 2004, 2012). Individual trace elements, macronutrients, and vitamins (thiamine, cyanocobalamine, and biotine) were from Merck.

Daphnids were fed with a concentrated suspension of the green algae *Pseudokirchneriella subcapitata*. The algae and the marine seaweed extract were supplied by Glenside Organics Ltd (UK). Woods Hole MBL growth medium supplied all macro- and micronutrients for the green algae *P. subcapitata*. The macronutrients used on MBL culture medium were calcium chloride dihydrate ($CaCl_2.2H_2O$), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), sodium bicarbonate ($NaHCO_3$), dipotassium phosphate (K_2HPO_4), sodium nitrate ($NaNO_3$), and sodium metasilicate pentahydrate ($NaSiO_3 \cdot 9H_2O$) from Prolabo (Belgium). The micronutrients ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$), sodium molybdate dihydrate ($NaMoO_4 \cdot 2H_2O$), and tris(hydroxymethyl)aminomethane were also from Prolabo.

Migration studies

Analytical procedure and method validation

BPA extraction was performed by solid phase extraction (SPE) using Strata SDB-L cartridges. After elution, the extracts were evaporated to dryness, reconstituted within ethyl acetate, and derivatized using MSTFA (Mansilha et al. 2012; Viñas et al. 2010).

Six BPA matrix-standard calibration solutions, at concentration levels ranging from 0.075 to 0.750 μg/L, were prepared just before the extraction procedure.

BPA-d_{16}, at a concentration of 0.750 μg/L, was used as procedural or instrumental internal standard for assessing the effectiveness of the extraction technique and monitoring the recovery during sample analysis due to a more or less complex sample preparation. Chromatographic analyses were carried out in a Shimadzu GCMS-QP2010 Gas Chromatograph Mass Spectrometer equipped with a fused-silica capillary column coated with 5 % diphenylmethylsiloxane, VF-5 MS (30 m×0.25 mm I.D., 0.25 μm film thickness) from Varian. Injections (1 μL) were made in the splitless mode with a 1.0-min purge-off time and injector temperature set at 275 °C. Calibration standards and water samples were analyzed using the following oven temperature program: initial temperature 60 °C (held for 2 min), increased by 10 °C/min to 200 °C (held for 1 min), increased again by 10 °C/min to 275 °C, and held at this temperature for 10 min. GC was directly interfaced to a Shimadzu QP 2010 quadrupole mass spectrometer with an interface temperature of 250 °C and ionization by 70 eV electron impact. The transfer line was set at 275 °C and the source at 200 °C (Mansilha et al. 2012).

Positive fragment ions (*m/z* ion mass/charge ratio) were analyzed over 43–500 *m/z* mass range in full scan mode and in selected-ion monitoring (SIM) mode.

The identification of BPA in the samples was done according the following conditions: (a) the relative or the absolute retention time of the sample component matched that of the authentic compound within a limit deviation of±0.02 min; (b) the selected diagnostic ions were present at the substance specific retention time; (c) the relative intensities of two of the chosen diagnostic ions measured in the sample do not deviate by more than 25 % (Stottmeister et al. 2009); and comparison with comprehensive mass-spectral libraries (such as NIST) that allowed an unequivocal identification of target compound.

The ions selected for quantification and identification purposes were mass-to-charge ratio (*m/z*): 386 (molecular ion, $[M]^{+\bullet}$), 369, 368, and 217 for BPA-d_{16} and 372 $[M]^{+\bullet}$, 357, and 217 for BPA with relative intensities of I_1 372 (100 %), I_2 357 (25 %), and I_3 217 (5 %).

Validation was done mainly according to the International Conference on Harmonization recommendations (ICH 2005), as well as to some European and American validation guidelines (EURACHEM 1998; NCCLS 2002; NCCLS 2004; SANCO 2009; USFDA 2001) with specifications for endocrine disrupting analysis and/or GC-MS methodology.

As the assumption of homoscedasticity was not met for analytical data, a weighted least squares linear

regression procedure was applied as a simple and effective way to counteract the greater influence of the higher concentrations on the fitted regression analysis, improving accuracy at the lower end of the calibration curve (Miller and Miller 2005).

Several parameters such as: specificity and selectivity, linearity and linear range, limits of detection and quantification, precision, accuracy, trueness (recovery), stability, and robustness were determined and accessed.

Sampling plan

Twelve different brands of baby bottles of reusable PC, non-PC, and PP (polypropylene, commonly labeled "non-BPA" or "BPA-free"), an infant feeding cup, and three plastic containers used for food storage, two of PP and one of PC, were purchased in Oporto retail stores and used for high-temperature testing. Containers were filled with ultra-pure water and heated to 100 °C in a microwave. The boiling temperature was maintained for 1 min. Then the samples were left at room temperature for 2 h and, finally, were preconcentrated using SPE. High-temperature test was used because boiling the water in the microwave directly in baby bottles for sterilization purposes is a usual procedure when preparing formula. Also, the distribution of small amounts of hot soups and meals in small containers, which are then kept in the freezer for daily use, is frequent nowadays.

As baby bottles are typically used with their contents kept near body temperature, migration tests were also carried out at 40 °C for 240 h (USFDA 2007). This 10-day testing period was included in order to mimic repetitive use of baby bottles.

All containers were cleaned before use in a domestic dishwasher (55 °C) with exception of BB-7 (similar to BB-8) that was manually cleaned according to manufacturer's instructions to test dishwashing impact on BPA migration. Experimental planning is summarized in Table 1.

Biological tests

D. magna were obtained from continuous cultures maintained in the laboratory in ASTM hard water at a temperature of 20 °C±1 °C and a 16-h light:8-h dark photoperiod, at a light intensity of 100–1,000 Lx. Animal density was 15 individuals per 1,000 mL and medium was renewed three times a week. Daphnids were fed with algae (*Pseudokirchneriella subcapitata*) with a density of 5×10^5 cells/mL and an organic additive (Baird et al. 1989). Physical–chemical parameters as total hardness, oxygen concentration, pH, temperature, conductivity, and total organic carbon (TOC) were measured at least once a week, in fresh and old ASTM media, in the control(s) and in the test vessels.

Preliminary studies

Daphnids were subjected to a reference chemical test with potassium dichromate to ensure the validity of the experiments (ISO 2010). After that, acute tests were performed according to OECD 202 protocol (OECD 2004) as range finding tests. Young daphnids, aged less than 24 h at the start of the test, were placed in glass test vessels and exposed to BPA for a period of 48 h. Tests were conducted in glass vessels using nine concentrations of BPA (1.0, 5.0, 7.0, 9.0, 10.0, 11.0, 13.0, 15.0, and 20.0 mg/L) and untreated controls. Ten replicates per test concentration level and control were prepared. Immobilization (after 15 s of gentle agitation) was recorded at 24 and 48 h and was compared with the controls. The results were analyzed in order to calculate the EC_{50} at 48 h (concentration estimated to immobilize 50 % of the daphnids within a stated exposure period) as well as the lowest-observed effect concentration value (LOEC) and the no-observed-effect concentration value (NOEC).

The reproduction test was done according to the OECD 211 protocol (OECD 2012), whose primary objective is to assess the effect of chemicals on the reproductive output of *D. magna* and, to this end, young female *Daphnia* (the parent animals), aged less than 24 h at the start of the test, are exposed to the test substance added to water at a range of concentrations during 21 days. This long-term toxicity test allowed the examination of the survival of the parent animals, molting frequency, and the number of newborns produced. Three BPA concentrations were tested (one third of EC_{50} and two other concentrations above and below $EC_{50}/3$), using also ten replicates (females of *D. magna*) per concentration and control.

Multigenerational tests were also performed. Normally developed neonates (maximum age 24 h at the start of the test originated from the latest brood of daphnids from the reproduction test), which were exposed towards selected test concentrations during their embryonic development in mater, were further exposed to the same substance concentration. Neonates from unexposed control organisms were treated the same way and held in uncontaminated medium. Ten replicates were used for each BPA concentration and control.

Exposure tests. Breeding of D. magna in the plastic containers

After standardization of the methodologies, reproduction tests were carried in the plastic containers. In order to assess the biological impact of the BPA in real exposure conditions, individuals of *D. magna* were inserted and bred in several baby bottles and in other plastic containers. Two reproductive methodologies were applied using single and multigenerational tests.

Table 1 Containers used in the study and applied tests

Container brand	Vol (L)	Recycling symbol	Migration tests	Cost
Baby bottle				
BB-1	0.15	7:PC	High temperature (100 °C, 24 h); Repeated use (40 °C; 240 h)	Cheap
BB-2	0.15	7:PC	High temperature (100 °C, 24 h); Repeated use (40 °C; 240 h)	Cheap
BB-3	0.15	7:PC	High temperature (100 °C, 24 h); Repeated use (40 °C; 240 h)	Expensive
BB-4	0.40	7:PC	High temperature (100 °C, 24 h); Repeated use (40 °C; 240 h)	Expensive
BB-5	0.30	7:PC	High temperature (100 °C, 24 h)	Expensive
BB-6	0.20	7:PC	High temperature (100 °C, 24 h); Repeated use (40 °C; 240 h)	Expensive
BB-7	0.15	Non-PC	High temperature (100 °C, 24 h)	Cheap
BB-8	0.15	Non-PC	High temperature (100 °C, 24 h)	Cheap
BB-9	0.25	Non-PC	High temperature (100 °C, 24 h); Repeated use (40 °C; 240 h)	Cheap
BB-10	0.25	5:PP (BPA free)	High temperature (100 °C, 24 h)	Expensive
BB-11	0.25	5:PP (BPA free)	High temperature (100 °C, 24 h)	Expensive
BB-12	0.26	5:PP (BPA free)	High temperature (100 °C, 24 h)	Expensive
Infant feeding cup				
Cup-1	0.15	Non-PC	High temperature (100 °C, 24 h)	Cheap
Household plastic container				
C-2	0.50	5:PP	High temperature (100 °C, 24 h)	Cheap
C-3	0.25	5:PP	High temperature (100 °C, 24 h)	Cheap
C-4	0.50	7:PC	High temperature (100 °C, 24 h)	Expensive

Single generation reproduction test The single generation test was performed in ten baby bottles (BB-1, BB-2, BB-4, BB-9, BB-10, and five identical BB-8$_{(a\ to\ e)}$, used to monitor the reproducibility of the test), in the infant feeding cup and in the three household plastic containers. The ASTM culture medium was previously boiled at 100 °C, placed in each container and cooled to room temperature (18–22 °C). One *D. magna*, age under 24 h, was placed in each plastic test vessel. Tests were performed as described in OECD 211 guideline. During the test period, the frequency of female reproduction, survival, and the number of neonates were evaluated. The results were compared with those of the control glass beakers ($n=6$).

Multigenerational tests To verify the impact of BPA, and eventually of other plastic leached compounds, in several generations of *D. magna*, two reproduction tests were performed. First, five daphnids in different life stages (N0, N1, N2, N3, N4) were placed simultaneously in each test container (BB-2, BB-4, BB-9, BB-10, Cup-1, C-4) during 21 days. The frequency of female reproduction, survival, and the number of neonates was recorded.

Secondly, another toxicity study was performed over six generations during several months to evaluate the effects of long-term exposure of the daphnids. In this case, newly hatched neonates (F0) of the third brood from age-synchronized mothers were selected randomly and transferred individually into each tested container (BB-1, BB-2, BB-4, BB-8, BB-10, and C-3). Simultaneously, four neonates were also transferred to glass vials and used as control. All newly hatched neonates from each female were removed and counted, and after 21 days, females of the F0 generation were also removed and a single neonate per clutch was randomly selected as the F1 generation to continue the experiment in the plastic or glass container. Daphnids of subsequent generations were treated as in the F0 generation. Dead animals were not replaced. The experiment was finished after the neonates (F6) of the generation F5 had been hatched.

These studies were important to evaluate not only one generation of newborns but also the behavior of further generations, similarly to what happens in the environment. All multigenerational studies were performed in the same conditions described for single tests.

Data analysis

The effective concentration (EC$_{50}$ value) for the parameter immobilization was calculated using the results obtained in the 48 h acute toxicity tests. The dose–response curve was fitted via the probit model.

Data differences were tested for significance by means of one-way analysis of variance (ANOVA). When differences among groups were significant, the Dunnett's test was employed to compare the control group with each of

the experimental groups for calculation of NOEC and LOEC. Statistical significance was set at $p<0.05$, where the null hypothesis claims that there is no difference between samples and control growth rate. One-sample independent t tests and two-sided t tests, with a significance level of 0.05, were also carried out.

Results and discussion

Analytical method validation

Specificity and selectivity were evaluated by comparing the chromatograms of matrix-blank samples (samples of ultrapure water) with an aqueous solution of the silylated analyte at a concentration near the limit of quantification. No significant interference has been detected at the retention time of BPA. Selectivity was also assessed by the comparison of the analyte mass spectrum with a library spectrum.

Calibration in SIM mode was performed using external standardization. Matrix-calibration standards were prepared under the same conditions applied to samples and injected in triplicate. Regression parameters obtained after application of the weighting factors were calculated and are presented in Table 2. The random errors in the y direction ($S_{y/x}$), as well as the standard deviations for the slope (S_b) and intercept (S_a), were used to estimate confidence limits given by: $b\pm t_{(n-2)}S_b$ and $a\pm t_{(n-2)}S_a$, where the t value is taken at 95 % confidence level and ($n-2$) degrees of freedom. The percentages of $S_{(b)w/b}$ satisfy the validation condition of the calibration curve with a value less than 5 % (Miller and Miller 2005).

Good sensitivity (LOD 7.76 ng/L) was found under the optimized experimental conditions and the uncertainty for the calibration curve (U) that reflects the variability of the method was calculated according to: $U=Sx_{0w}/x_0$.

Intraday and interday precision results were in accordance with ICH, USFDA, and NCCLS requirements (ICH 1994, 1995; NCCLS 2002; USFDA 2001). Results were within 15 % (RSD), except at the LOQ, which assume a value of ±20 %. Accuracy was expressed as absolute bias and was inferior to ±15 % of the accepted true value (ICH 1995).

Instrumental precision was evaluated and presented an acceptable value. Media and amplitude control charts of BPA-d$_{16}$, used for process evaluation through recovery

studies, showed no relevant tendency, so no procedural review was necessary (Mansilha et al. 2012).

Migration of bisphenol A from plastic containers

Table 3 summarizes the results of the high temperature migration tests showing, unequivocally, that BPA migration from PC containers is higher when compared to other plastic materials. This is in agreement with other studies that documented that BPA migration increase at higher temperatures due, probably, to the hydrolysis of the PC polymer or to the liberation of non-reactive monomers (Kubwabo et al. 2009).

Results have also shown that there is no relationship between BPA concentrations and the price of the product, but only with the polymers they are made of. BB-10, BB-11, and BB-12, labeled as *BPA Free*, as well as C-2 and C-3, showed only residual levels of BPA. This confirms that 5:PP containers may be considered an appropriate alternative to the PC ones in order to minimize BPA exposure.

The impact of dishwashing on BPA migration was also investigated. Two identical baby bottles were tested simultaneously for two different wash procedures. BPA concentration in the sample collected from the bottle washed in the dishwasher (BB-8) was 0.34 µg/L; while in the manual cleaned bottle (BB-7), a significantly lower value of 0.06 µg/L was obtained ($p<0.05$).

The 10-day test was designed to simulate repeated use and to investigate the highest concentration levels that could potentially migrate from the materials used to fabricate reusable baby bottles at a temperature near body temperature. Six brands of baby bottles (BB-1, BB-2, BB-3, BB-4, BB-6, and BB-9) were filled with water and incubated for 240 h (10 days) at 40 °C. BB-5, as well as BB-7 and BB-8, were not tested as their high temperature test results were similar to BB-3 and to BB-9, respectively. The "BPA free" baby bottles (BB-10, BB-11, and BB-12) were also not processed as they revealed no significant BPA levels. Residual BPA ranged from 1.83 to 5.63 µg/L with an average of 3.75 µg/L. Comparing these results with those obtained for the same six baby bottles in the high-temperature migration test, an average BPA concentration value of 6.13 µg/L was achieved. So, BPA seems to be more readily released (1.6 times) at higher temperatures.

Table 2 Method validation data

	$b_w\pm t_{(n-2)}\cdot S_{(b)w}$ (95 %)	$a_w\pm t_{(n-2)}\cdot S_{(a)w}$ (95 %)	$S_{(y/x)w}$	$S_{(b)w}/b_w$ (%)	r_w	LOD (ng/L)	LOQ (ng/L)	$U_{0.075\ ppb}$ (%)	$U_{0.750\ ppb}$ (%)
Bisphenol A	1,500.41±19.41	−803.23±5,808.37	2,588.30	0.47	0.9998	7.76	25.88	3.97	1.13

Calibration parameters and related uncertainties

Table 3 Results of BPA levels obtained for plastic containers tested at high temperature

Container brand	Recycling symbol	BPA concentration (μg/L)
Baby bottle		
BB-1	7:PC	3.99
BB-2	7:PC	10.3
BB-3	7:PC	1.45
BB-4	7:PC	15.2
BB-5	7:PC	1.02
BB-6	7:PC	5.54
BB-7	Non-PC	0.06
BB-8	Non-PC	0.34
BB-9	Non-PC	0.28
BB-10	5:PP (BPA free)	0.04
BB-11	5:PP (BPA free)	<LOQ
BB-12	5:PP (BPA free)	0.05
Infant feeding cup		
Cup-1	Non-PC	0.08
Household plastic container		
C-2	5:PP	0.04
C-3	5:PP	0.05
C-4	7:PC	0.26

Biological tests

Physical–chemical parameters of the ASTM hard water were measured during the tests. The following values were obtained: total hardness 201 ± 5 mg/LCaCO$_3$, oxygen concentration 9.6 ± 0.8 mg/LO$_2$, pH range 7.0–8.0, TOC<2 mg/L, and conductivity 480 ± 15 μScm^{-1}.

Preliminary studies

The value of EC$_{50}$ obtained in 24 h for cultures of *D. magna* exposed to potassium dichromate was 1,400 μg/L (±50 μg/L), which is within the range stipulated by ISO 6341 (EC$_{50}$ 600 to 1,700 μg/L). The acute toxicity test result, expressed as EC$_{50}$, was 9940 μg/L (±130 μg/L) after 48 h of exposure. This value was in agreement with those reported by other authors (1,000 to 20,000 μg/L; Alexander et al. 1988; Brennan et al. 2006; Caspers 1998; Mu et al. 2005; Staples et al. 1998), confirming that the stipulated laboratory conditions were successfully applied. Based also on the acute test data, the LOEC was determined to be 5,000 μg/L resulting in an NOEC of 3,000 μg/L. These results were in accordance with those reported on the European Union Risk Assessment Report published on 2003 (EU 2003).

The reproduction toxicity test was processed during 21 days for three different concentrations of BPA (1,000, 3,000, and 5,000 μg/L) plus control. BPA concentrations were determined weekly and were satisfactorily maintained within ±20 % of the nominal concentration. Neonates from each replicate were counted and removed from the test vessels daily after the appearance of the first brood.

No adult mortality occurred in the control vessels over the 21-day testing period. The mean number of live offspring produced per control parent animal was 61 and the minimum number was 60; thus, this test was valid according to the OECD guideline 211 biological criterions.

Daphnids in BPA concentration of 5,000 μg/L died after 14 days. In 1,000 and 3,000 μg/L, results demonstrated that after the 21-day trial the number of newborns per live adult obtained in control and in BPA tests underwent no significant differences ($p\leq0.05$). These results were consistent with the acute test results and with the study of Staples et al. (1998), in which a concentration of 3150 μg/L of BPA promoted no relevant effects. However, it should be noted that it was observed a precocious maturity in the concentration of 3,000 μg/L of BPA when compared to control (Fig. 1). Caspers also found an accelerating effect of BPA on offspring production (Caspers 1998).

So, in this study, no statistically significant differences were observed, at a significance level of $\alpha=0.05$, between the total live offspring per survival female of the control batch and the two BPA batches (1,000 and 3,000 μg/L). However, at 3,000 μg/L, an early enhancement was observed between days 6 and 13.

Multigenerational tests were also performed for the same concentrations of BPA. The exposure conditions and the applied procedures were similar to those of the reproduction toxicity test.

Overall fecundity (mean neonates per female over 21 days) was not affected in F0 (1st brood) at 1,000 and 3,000 μg/L treatment levels; but in F1 (2nd brood) and F2 (3th brood), fecundity was significantly affected by BPA ($p<0.05$) and a precocious maturity was also recorded. In F3 (4th brood), an enhancement was observed in 1,000 μg/L, although in 3,000 μg/L daphnids died after 16 days. F4 daphnids (5th brood) died on the 8th day in 1,000 μg/L BPA test (Fig. 2).

Molting behavior and survival are toxicological endpoints which have been shown to be adversely affected by this endocrine disrupting chemical. Data analysis demonstrates the effects of BPA on the survival and reproduction over generations, providing insight into the relative sensitivity and higher biological level consequences of BPA exposure.

Exposure tests. Breeding of D. magna in the plastic containers

Single generation chronic test Single generation reproduction toxicity test was performed during 21 days in 10 baby bottles, in the feeding cup, and in some household plastic

Fig. 1 Mean cumulative number of neonates produced by *Daphnia magna* exposed to various BPA concentrations and control over the time course of the experiment (*n*=10)
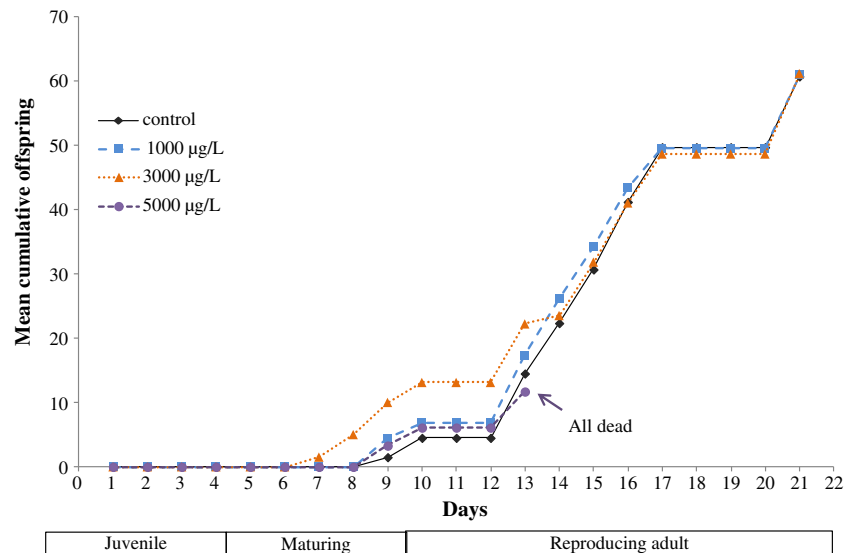
containers. Six glass beakers were used as negative control and a confidence interval (CI) of ±1.9 was calculated with a level of significance of 0.05. A one-tailed *t* test was then applied in order to check if the *D. magna* offspring in plastic containers was significantly different from the control. A significantly higher offspring number was observed in all samples, except in Cup-1, C-2, and C-3, when compared with control. The results achieved with the five baby bottles BB-8 shown the reproducibility of the test (Fig. 3), with offspring numbers between 83 and 86 and a confidence level of ±1.4 (α=0.05). Data analysis also demonstrated a positive correlation between BPA concentrations obtained in the high temperature test (100 °C) and the offspring numbers (r^2=0.97).

Multigenerational chronic tests As daphnids undergo distinct life stages during their development, which may be affected differently by toxicant pulses, a first multigenerational test was performed in six of the plastic containers used in single test (BB-2, BB-4, BB-9, BB-10, Cup-1, C-4). There were used immature juveniles (0–5 days old—N0 and N1), organisms approaching sexual maturity (5–10 days old—N2 and N3) and reproducing adults (10–15 days old—N4) in each studied container. Data are also showed in Fig. 3. Three glass beakers were used as control and a CI of ±2.0 was calculated with a level of significance of 0.05. A one-tailed *t* test was performed. It was observed a significant higher offspring in BB-2 and BB-4, which were PC baby bottles, as expected regarding single test results. There was also a positive correlation between BPA concentration and the offspring numbers (r^2=0.83).

These experiments demonstrated that BPA seems to disrupt the reproductive process, both in single and in

Fig. 2 Multigenerational chronic test. Effects of BPA on brood sizes over five generations of *D. magna*

*   Died on 8th day
**   Died on 16th day
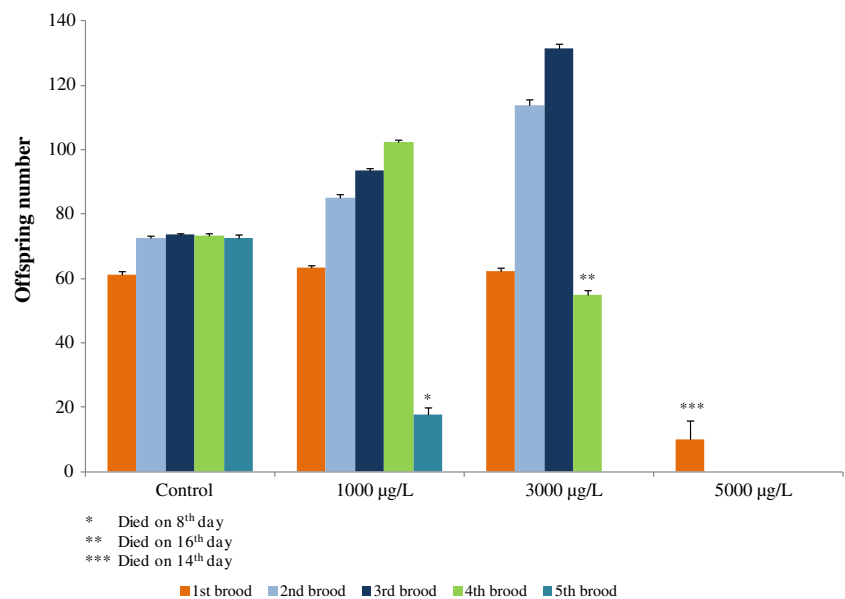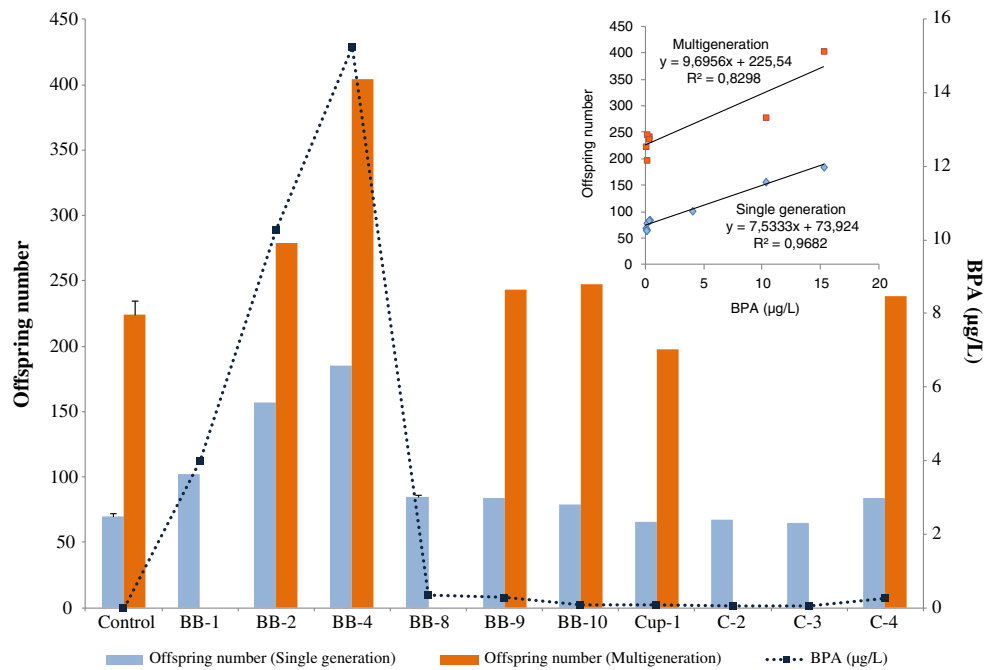***   Died on 14th day

Fig. 3 Correlation between BPA migration results and single and multigenerational offspring numbers in the 21-day test

multigenerational tests, resulting in the production of more neonates when compared with controls. However, this conclusion contrasts with the previous studies with defined BPA concentrations (Fig. 1) that showed no significant impact of BPA at 3,000 μg/L.

A second multigenerational study, performed over six generations, showed also a significant reproduction enhancement, especially in BB-2 and in BB-4, but also in BB-1 and BB-8, when compared with control.

As in single generation test, there were used five identical baby bottles BB-8 to assess the reproducibility, or the uncertainty, associated with the experiment and results showed no significant differences regarding offspring numbers for all generations, with confidence levels varying from 0.6 to 2.2, except for F5 were offspring numbers varied from 44 to 69 (confidence level 14.4).

In this study, it was observed that offspring numbers were almost constant over all generations for each baby bottle until F4 (5th brood). F5 daphnids (6th brood) died in BB-1, BB-2, and BB-4, demonstrating the effects of the exposure on parental mortality. As expected, in C-3 the results did not differ from control (Fig. 4). Daphnids exposed to higher BPA concentrations also matured faster than control animals in all generations.

It seems that breeding the crustacean *D. magna* in the plastic containers resulted in an increased reproductive output of daphnids and in the decrease of parental survival after five generations, especially for those cultured in PC bottles where higher concentrations of BPA were detected. Although these concentrations were far below than those used in defined concentrations testes, we can conclude that direct exposure

to plastics produced evident ecotoxicological effects. These results could indicate a non-monotonic dose–response relationship, which is the basis of the "low dose" concept. The pharmacokinetics of low doses of bisphenol A has been discussed in the last years and there are already several studies on this subject. However, important knowledge data gaps have remained largely unaddressed. So, in connection with the efforts to characterize the risks associated with EDCs, it has been argued that the current risk assessment paradigm needs modification or has become obsolete because EDCs elicit effects at doses much lower than normally used in regulatory testing (Kortenkamp et al. 2012). Another plausible explanation for the observed results could be the existence of a mixture of leached substances in solution, since it is known that along with BPA, plastics release other compounds such as phthalates that are also considered endocrine disruptors (González-Castro et al. 2011; Guart et al. 2011; National Research Council 2008). Although it is recognized that organisms face multifarious mixtures of environmental stressors rather than individual chemicals at set doses and that the effects of a mixture can be very different from the sum of the contributions of the individual compounds, until now little attention has been paid to the simultaneously interaction of different pollutants and to cumulative risks, firstly highlighted in the *Framework for Cumulative Risk Assessment* of USEPA (2003). This led to the concern that testing of single chemicals may completely underestimate effects in mixtures, leading to the erroneous conclusion that no effects are observed, whereas in fact, effects would be expected in the field environment where animals

Fig. 4 Correlation between BPA migration results and the offspring numbers of *Daphnia magna* bred in different plastic containers and in control vessels over six generations
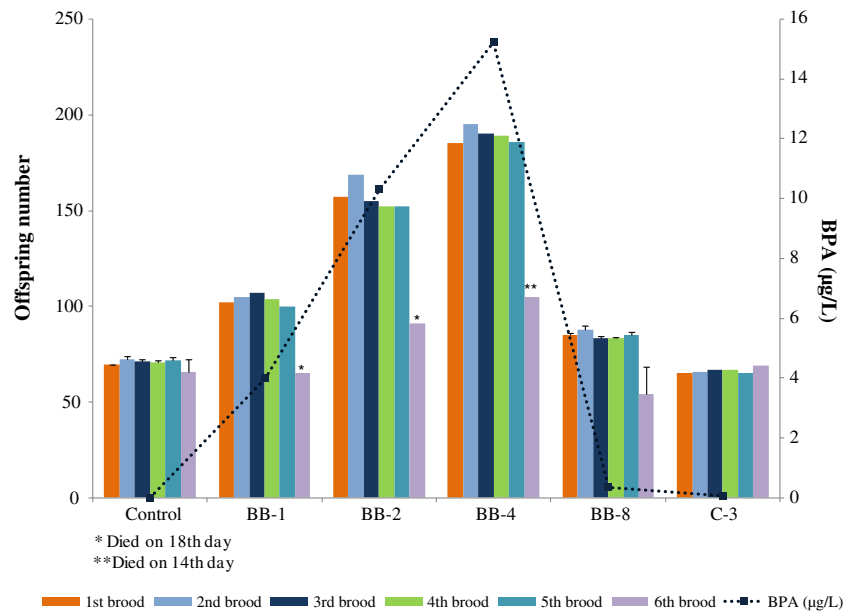
are exposed to mixtures (Kortenkamp et al. 2012; Loureiro et al. 2010; Barata et al. 2006).

Conclusions

An analytical method for the assessment of BPA migration from plastic containers was validated according to international conventions and allowed the quantification of very low levels of BPA. The migration of BPA from several baby bottles and other plastic containers revealed, in some cases, a significant release of BPA indicating that a broader range of foodstuff may be contaminated with endocrine disruptors when packed in plastics.

Ecotoxicological acute test results were similar to those reported by other authors and the reproduction test showed no significant differences comparing to control, although it was observed a precocious maturity in the concentration of 3,000 μg/L. Additionally, single and multigenerational tests were conducted using some containers previously tested concerning BPA migration. Culturing of *D. magna* in plastic containers filled with defined culturing medium led to a significant increase of its reproductive output (neonates per female) in all PC brands tested (145.1±4.3 % to 264.7±3.8 %), both in single as in multigenerational tests, in comparison with the negative control group (100.3±1.6 %). In contrast, neonate production by daphnids cultured in PP and non-PC bottles was slightly, but not significantly, enhanced (92.5± 2.0 % to 118.8±1.8 %).

Interesting results were obtained as low-dose-dependent ecotoxicological effects on *D. magna* were observed, i.e., in the plastic containers, the BPA migration values were positively correlated with the offspring

numbers at concentrations far below 3,000 μg/L (the NOEC calculated value). The positive correlation results obtained allowed establishing a quantifiable approach and a prediction of the toxicity of a chemical mixture from the results of a single substance as already reported by other authors (Ashauer et al. 2006). The results also illustrated the importance of transgenerational studies, since if the test was discontinued after the first generation, a different conclusion about the toxicity of BPA on the fertility of *D. magna* would be obtained. A magnification of the adverse effects on mortality and fecundity has been observed, which represents a worrying trend for organisms that are chronically exposed to xenoestrogens for many generations (Brennan et al. 2006).

This study demonstrate that the ecotoxicological effects resulting from the exposure to defined concentrations of a substance could be different from those obtained in more realistic exposure conditions, which can lead to very different values of NOEC and LOEC that have been criticized, in the last years, regarding regulatory aspects (Kortenkamp et al. 2012; Warne and van Dam 2008). Therefore, from a legislative point of view, and according the Final Report of the State of the Art Assessment of Endocrine Disruptors, it is of great importance to have information about the spectrum of EDCs that are present in relevant exposure scenarios, as this information is currently fragmentary, and this lack of information makes it likely that the full extent of risks associated with EDCs might be underestimated (Kortenkamp et al. 2011). Thus, a targeted testing strategy for "low-dose" experiments and the selection of mixtures of compounds to be tested along with BPA, reflecting more realistic scenarios, is necessary and requires further investigation for a more accurate risk assessment.

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