Bisphenol A Is Released from Used Polycarbonate Animal Cages into Water at Room Temperature

Kembra L. Howdeshell,[1] Paul H. Peterman,[2] Barbara M. Judy,[3] Julia A. Taylor,[3] Carl E. Orazio,[2] Rachel L. Ruhlen,[1] Frederick S. vom Saal,[1] and Wade V. Welshons[3]

[1]Division of Biological Sciences, University of Missouri, Columbia, Missouri, USA; [2]U.S. Geological Survey, Columbia Environmental Research Center, Columbia, Missouri, USA; [3]Department of Veterinary Biomedical Sciences, University of Missouri, Columbia, Missouri, USA

Bisphenol A (BPA) is a monomer with estrogenic activity that is used in the production of food packaging, dental sealants, polycarbonate plastic, and many other products. The monomer has previously been reported to hydrolyze and leach from these products under high heat and alkaline conditions, and the amount of leaching increases as a function of use. We examined whether new and used polycarbonate animal cages passively release bioactive levels of BPA into water at room temperature and neutral pH. Purified water was incubated at room temperature in new polycarbonate and polysulfone cages and used (discolored) polycarbonate cages, as well as control (glass and used polypropylene) containers. The resulting water samples were characterized with gas chromatography/mass spectrometry (GC/MS) and tested for estrogenic activity using an MCF-7 human breast cancer cell proliferation assay. Significant estrogenic activity, identifiable as BPA by GC/MS (up to 310 µg/L), was released from used polycarbonate animal cages. Detectable levels of BPA were released from new polycarbonate cages (up to 0.3 µg/L) as well as new polysulfone cages (1.5 µg/L), whereas no BPA was detected in water incubated in glass and used polypropylene cages. Finally, BPA exposure as a result of being housed in used polycarbonate cages produced a 16% increase in uterine weight in prepubertal female mice relative to females housed in used polypropylene cages, although the difference was not statistically significant. Our findings suggest that laboratory animals maintained in polycarbonate and polysulfone cages are exposed to BPA via leaching, with exposure reaching the highest levels in old cages. *Key words:* animal caging, bisphenol A, endocrine disruptor, estrogen, leaching, polycarbonate, polysulfone. *Environ Health Perspect* 111:1180–1187 (2003). doi:10.1289/ehp.5993 available via *http://dx.doi.org/* [Online 5 February 2003]

Bisphenol A (BPA) is a monomer used in the manufacture of many types of products, including polycarbonate plastic food storage containers (i.e., baby bottles and water carboys), as well as the epoxy resin used as the lacquer lining of food or beverage cans and in some dental sealants. Exposure to the monomer has been reported to occur as a result of the migration of BPA out of these products when they are exposed to high heat or alkaline conditions (Brotons et al. 1995; Olea et al. 1996). Krishnan et al. (1993) reported that polycarbonate flasks leached BPA into media in the autoclaving process. Likewise, BPA is known to be released from reusable polycarbonate baby bottles as a result of washing (Consumers Union 1999). Recent research in Japan has shown that leaching of BPA from polycarbonate products increases as a function of the amount of wear on the product because of repeated use (Takao et al. 1999). There is considerable interest in whether use of BPA in food and beverage containers as well as in other products can result in significant release of BPA, because BPA has been shown to be estrogenic in both *in vitro* cell culture (Krishnan et al. 1993; Nagel et al. 1997) and *in vivo* rodent assays. This concern is increased by findings that levels of BPA detected in human fetal umbilical cord blood (Schönfelder et al. 2002b) are within the range of effects in studies with rats and mice.

Polycarbonate animal cages are commonly used to house laboratory animals, such as rodents and aquatic invertebrates and vertebrates, because of the assumption that these cages have superior heat-resistant properties and structural strength. This assumption is also typically made with regard to other polycarbonate products, such as baby bottles. Because of their presumed durability, the polycarbonate cages are generally used long after they show significant signs of wear. Continued use of discolored, clearly worn cages may occur because the plastic cages (and other plastic products) are commonly assumed to be manufactured from highly stable polymers. The objective of our study was to examine this assumption by observing whether used polycarbonate animal cages would release free BPA (unreacted BPA monomer or BPA from hydrolysis) under normal use conditions of room temperature (23 ± 2°C) and a neutral solvent, high-pressure liquid chromatography (HPLC)-grade water (pH 7). These experimental conditions are directly relevant because it is common for aquatic laboratory animals to be housed in polycarbonate cages. However, the potential for effects of BPA leaching from polycarbonate products on rodents cannot be discounted because effects at very low doses (micrograms per kilogram per day) of BPA have been reported (Elswick et al. 2000; Farabollini et al. 1999; Gupta 2000; Honma et al. 2002; Howdeshell et al. 1999; Markey et al. 2001a; Nagel et al. 1997; Palanza et al. 2002; Rubin et al. 2001; Sakaue et al. 2001; Schönfelder et al. 2002a; Steinmetz et al. 1998; vom Saal et al. 1998)

We also evaluated new polycarbonate cages as well as new polysulfone cages, another type of plastic manufactured from BPA. Polysulfone is marketed as having a higher temperature and chemical tolerance than polycarbonate cages and thus may be less likely to leach BPA. The bioactivity of the cage water samples was tested in an *in vitro* cell proliferation assay using estrogen-sensitive MCF-7 human breast cancer cells to determine whether the BPA measured by gas chromatography/mass spectrometry (GC/MS) was sufficient to elicit a biological response in human breast cancer cells. Finally, the *in vivo* estrogenic bioactivity of the used polycarbonate cages was tested by measuring the uterine wet weight of prepubertal female mice housed in the cages.

Materials and Methods

Animal caging. New and used polycarbonate rodent cages (Allentown Caging Equipment, Allentown, NJ) were obtained from two different animal care facilities at the University of Missouri. The new polycarbonate cages had not been used to house animals, whereas the used polycarbonate cages that were selected were visibly worn, with patches of opaque plastic and some areas of rough, pitted surface inside the cage. New polysulfone cages (Allentown Caging Equipment) were also tested. Glass casserole dishes were used as negative controls because they do not contain any plastic products. Used polypropylene rodent cages (Lab Products, Inc., Maywood, NJ) were selected as a negative control caging material because they are not manufactured with BPA. All plastic cages were standard size

(29 × 19 × 13 cm) except the two new polycarbonate cages, which measured 29 × 19 × 13 cm with a wider base (cage 1) and 32 × 24 × 15 cm (cage 2).

Cage leaching experiment. We examined the leaching of BPA from new and used polycarbonate cages as well as new polysulfone cages, used polypropylene cages, and glass containers in three replicate experiments. Before beginning each experiment, the cages and glass dishes were lightly rinsed with cold tap water to remove dust and any residual detergent that might be on the cages from washing in a sanitizing industrial cage washer. Subsequently, the cages and glassware were lightly rinsed twice in HPLC-grade water to remove any residue from the tap water. The rinsing procedure also should have removed free BPA from the cage surface. The plastic cages were not washed in the industrial cage washer between replicate leaching experiments. All glassware (casserole dishes, graduated cylinders, and collection tubes and flasks) used in the first leaching experiment was rinsed with 100% ethanol and allowed to dry before use. All glassware in the replicate leaching experiment was rinsed in 100% methanol and allowed to dry before use. The aluminum foil was rinsed with 100% methanol to remove potential contamination, such as phthalates, and allowed to dry before being used to cover the cages and dishes.

To test for leaching of BPA, we added a 250 mL volume of HPLC-grade water (Fisher Scientific, St. Louis, MO) to each of the plastic rodent cages. The surface area covered by the water varied slightly depending on the inner dimensions of the cages: polypropylene cages, 428 cm^2; used polycarbonate and new polysulfone cages, 446 cm^2; and the new polycarbonate cages, 450 cm^2 (cage 1) and 495 cm^2 (cage 2). Each glass dish was filled with 150 mL of HPLC-grade water; the water in the glass dishes covered a surface area of 222 cm^2. The volume of water added to the cages and the glass dishes was based upon the amount of water necessary to fill a standard size cage (29 × 19 × 13 cm) to a 2.5-cm depth, which is similar to the depth of corn cob bedding in a mouse cage. The surface area was determined by using a measuring tape on the interior surface of the glass dishes or cages. The top of each cage and dish was covered with aluminum foil to prevent dust contamination and evaporation of the water. The cages and glass dishes containing water were left undisturbed for 1 week at room temperature (23 ± 2°C).

After 1 week, the water samples were collected and analyzed to determine whether BPA had leached from the polycarbonate cages or was present in any of the negative control materials. An average volume of 222 ± 4 mL of the cage water (or 127 ± 5 mL of the glass dish water) was placed in 250 mL extraction flasks

for the analysis of BPA content by GC/MS. Two of the used polycarbonate cages (cages 3 and 4) leaked the HPLC-grade water during the week-long incubation. Therefore, the volume of water analyzed by GC/MS was less (cage 3, 108 mL of a final volume of 143 mL; cage 4, 195 mL of a final volume of 225 mL); the amount of BPA leached per surface area was calculated based on the volume of water remaining in the cages at the end of the week incubation. Two 10-mL aliquots of each of the cage or glass dish water samples were stored in methanol-rinsed test tubes with Teflon-coated caps. These samples were used in the MCF-7 human breast cancer cell proliferation assay.

GC/MS analysis. A 220 mL volume of each cage water sample, or 130 mL of each glass dish water sample, was acidified (pH 3) with HCl, extracted with dichloromethane in a separatory funnel and reduced with nitrogen to 0.1 mL, and analyzed by full-scan (*m/z* 34–700 electron ionization at 0.75 sec/scan) GC/MS using a ThermoFinnigan CE 8000 Top GC/Voyager quadrupole MS system (ThermoFinnigan, San Jose, CA). Each water sample was initially spiked with a stable-labeled $^{13}C_{12}$-BPA surrogate (at 1,000 ng in 10 µL; Cambridge Isotope Labs, Andover, MA) to monitor the efficiency of the extraction process, enhance the chromatography of this polar compound, and facilitate accurate quantitation of BPA. Likewise, an instrumental standard of deuterated (D_{14})-*p*-terphenyl (50 ng; AccuStandard, New Haven, CT) was added to the vial of each sample extract before injection into the GC/MS to verify instrument performance. Compounds in the water sample extracts were resolved using a 50-m Ultra-2 capillary column (0.20 mm inner diameter × 0.11 µm outer diameter; Agilent Technologies, Wilmington, DE) that was temperature programmed from 140°C to 300°C at 3°C/min. Automated, cool-on-column injections (2 µL) were made directly into the column's 2.5 m × 0.53 mm inner-diameter retention gap. To assure accuracy over the wide concentration range, a full quantitation calibration curve was analyzed via isotope dilution method using seven levels of BPA (32–12,700 ng in 200 µL) relative to ^{13}C-BPA (1,000 ng). For quantitation, two ion response factors were used for comparison, *m/z* 213:*m/z* 225 (base peaks of BPA and surrogate, respectively) and *m/z* 228:*m/z* 240 (molecular ions of BPA and surrogate). The detection limit was approximately 0.2 ng BPA on column with 10 ng of ^{13}C-BPA coeluting. Method detection limit was approximately 0.05–0.1 µg/L. Although BPA was specifically targeted for analysis, the full-scan GC/MS analysis was chosen to detect other possible estrogen-mimicking contaminants, including phthalates and nonylphenol.

MCF-7 cell proliferation assay. MCF-7 cells, an estrogen-sensitive human breast cancer cell line, were originally obtained from

V. C. Jordan, University of Wisconsin-Madison. The cells were cultured in maintenance medium [minimal essential medium (MEM) with nonessential amino acids, 10 µg/mL phenol red, 10 mM HEPES, 6 ng/mL insulin, 100 U/mL penicillin, 100 µg/mL streptomycin, and 5% charcoal-stripped calf serum] at 37°C and 5% CO_2.

One 10-mL aliquot of each water sample was dried down completely under gaseous N_2. Each water sample was then reconstituted with 100 µL of methanol and 14 mL of media. The cells were seeded (10,000 cells/well) in 24-well plates on day 0 in phenol red–free (estrogen-free) maintenance medium. They were fed on day 1 with the same medium and treated for days 3–6 with the media containing the reconstituted water samples, a range of 17β-estradiol (0.1–100 pM), or 0.1 mM LY156758 (an antiestrogen, also called keoxifene), with daily medium changes. All treatments were run in triplicate wells. On day 7, the wells were washed with 1 mL of Hanks' balanced salt solution (HBSS) with 25 mM HEPES, and each well was assayed for DNA content according to the method of Labarca and Paigen (1980). Calf thymus DNA was used as a standard after calibration by absorbance at 260 nm, assuming 20 absorbance units for 1 mg DNA/mL.

Materials for the cell proliferation and DNA assays were purchased from the following sources: MEM with nonessential amino acids (powdered), bovine calf serum, and lyophilized trypsin were obtained from Gibco/BRL (Grand Island, NY). HEPES, bovine insulin, calf thymus DNA type I, Hoechst dye 33258, streptomycin sulfate, penicillin-G, EDTA, HBSS, phenol red (sodium salt), and 17β-estradiol, were obtained from Sigma Chemical Co. (St. Louis, MO); all were "cell culture tested" when available. The antiestrogen LY156758 (keoxifene) was a gift from Eli Lilly and Company (Indianapolis, IN).

HPLC analysis. One of the cage water samples that possessed estrogenic activity in the cell proliferation assay and one of the negative control water samples were then processed by HPLC following the method described in Grady et al. (1991). The HPLC fractions (0.5 min/fraction) were dried down and reconstituted as above, and then applied to the cell proliferation assay. The objective was to identify which of the chromatographic fractions were estrogenic, and whether they could be excluded from being BPA or other specific estrogenic compounds, based upon their elution time. This approach measures each fraction regardless of whether there is an identifiable peak by ultraviolet spectrometry, because the MCF-7 bioassay is much more sensitive than the ultraviolet detector. The cell proliferation assay on the extracted fractions after HPLC was performed as described above, using 96-well instead of 24-well culture plates.

The sensitivity of the HPLC/bioassay was approximately 20 ng for BPA, 2 ng for genistein, and 0.5 pg for estradiol.

Animals and housing conditions. CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA) in 1998 and were then maintained as a closed outbred colony at the University of Missouri-Columbia. All mice were maintained at 23 ± 2°C under a 12:12-hr light:dark cycle. Breeder pairs of adult male and female mice were maintained in standard polypropylene cages (29 × 19 × 13 cm) on corncob bedding. Breeding pairs were fed Purina 5008 (soy-based; Ralston-Purina, St. Louis, MO) breeder chow, and water was provided *ad libitum* in glass bottles; water was purified by ion exchange followed by a series of carbon filters. At weaning (postnatal day 19), the females were maintained on Purina 5001 (soy-based) standard chow and provided purified water (as previously described) *ad libitum* in either glass or used polycarbonate bottles as specified below. Animals were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and all procedures were approved by the University of Missouri Animal Care and Use Committee.

Prepubertal mouse uterotrophic assay. On postnatal day 19, six females per litter were weaned and placed in one of two housing conditions, such that each litter was represented in each housing condition. Three females per litter were placed in used polycarbonate cages with corncob bedding and provided purified water in used polycarbonate bottles. The remaining three female siblings were housed in polypropylene cages with corncob bedding and provided purified water in glass bottles. We selected females from litters with 8–15 pups to reduce differences in body weight at weaning due to litter size. For litters with only three females each, we housed the three female siblings together and equally distributed these litters between the two cage types (polypropylene, $n = 3$ litters; used polycarbonate, $n = 3$ litters). Another litter with only five females was combined with a litter with only one female, such that three females siblings were placed in a used polycarbonate cage and the remaining two females siblings were housed in a polypropylene cage with the lone female from the other litter. We conducted this experiment in three replicates of approximately six litters per cage type, for a total of 57 animals per cage type. The total number of litters represented in each cage type was 19 litters in used polycarbonate cages and 20 litters in used polypropylene cages.

After 1 week (postnatal day 26), the body weight of the female mice was recorded. The females were euthanized with CO_2, their uteri were removed, and uterine wet weights were recorded. The 7-day exposure period was selected because group-housed CD-1 female mice do not show evidence of uterine growth and ovulation indicative of the completion of puberty at 26 days of age, when kept separate from males and maintained on the Purina 5008/5001 feeds (Howdeshell KL. Unpublished observations; Vandenbergh 1989). Uterine weight gain due to stimulation by estrogenic chemicals leaching from the used polycarbonate cages and water bottles could thus be compared with uterine weight gain in females housed in polypropylene cages with glass water bottles.

Statistical analysis. The analyses of the prepubertal mouse uterotrophic assay data were conducted using the Statistical Analyzing System (SAS) General Linear Model procedure (SAS Institute, Inc., Cary, NC). The uterine wet weight data were log-transformed to achieve homogeneity of variance, and then analyzed by analysis of covariance (ANCOVA) to determine whether organ weight measures needed to be corrected for body weight. Because body weight accounted for a significant component of the variance in uterine weight, uterine weight data were adjusted for body weight by ANCOVA. In addition to correcting for body weight, we also corrected for litter (maternal) effects. To control for litter effects, litter was entered as a main effect, and the *F*-value for cage type was divided by the *F*-value for litter. The comparisons of differences between group means were made using Fisher's least squared means test in SAS and, as mentioned above, the means for these analyses were all adjusted for body weight and litter effects. The confidence level for rejecting the null hypothesis was $p < 0.05$.

Results

Cage leaching experiment. We conducted three experiments to determine whether BPA leached out of different types of animal caging under neutral conditions by using HPLC-grade water as the solvent and at room temperature (23 ± 2°C). We did not use any materials, such as brushes, to scratch the surface, or movement to accelerate the release of BPA. Thus, we determined the passive release of BPA and other chemicals from these cages. The resulting water samples were extracted and analyzed by GC/MS for the detection of BPA.

The first leaching study was conducted to determine if used polycarbonate cages leached a detectable amount of BPA. The study included two each of used polycarbonate cages, used polypropylene cages, and glass dishes. The two used polycarbonate cages leached 110 and 51 µg BPA/L water, or 62 and 29 ng/cm² BPA per surface area, respectively (Table 1). The water samples from the negative control glass dishes and polypropylene cages did not contain detectable BPA.

We then conducted two additional leaching experiments, which included evaluating whether new polycarbonate cages also leached a detectable level of BPA under the same neutral conditions. The second leaching experiment included the same used polycarbonate cages and negative controls from the first experiment, with the addition of two new polycarbonate cages. A third leaching experiment included all the cages from the first and second leaching studies, plus two new polysulfone cages (also manufactured with BPA) and two additional used polycarbonate cages. The additional used polycarbonate cages were similar in appearance to the previously examined used polycarbonate cages.

Although a small amount of BPA (0.3 µg/L) migrated out of the new polycarbonate cages, the used polycarbonate cages released much higher concentrations of the chemical than did the new polycarbonate cages (Table 1). The BPA concentration in the water from only one of the two new polycarbonate cages could be measured in the second leaching study because of the poor recovery of the ^{13}C-BPA surrogate

Table 1. Concentration of BPA in cage water samples.

Cage description	Amount of BPA leached per surface area (ng/cm²)			Concentration of BPA per sample (µg/L)		
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
Glass dish						
1	< 0.07 LQ	—	< 0.13 LQ	< 0.1 LQ	—	< 0.2 LQ
2	< 0.07 LQ	—	< 0.13 LQ	< 0.1 LQ	—	< 0.2 LQ
Polypropylene cage						
1	< 0.03 LQ	—	< 0.12 LQ	< 0.05 LQ	—	< 0.2 LQ
2	< 0.03 LQ	—	< 0.12 LQ	< 0.05 LQ	—	< 0.2 LQ
Used polycarbonate cage						
1	62	10	10	110	18	18
2	29	23	12	51	41	22
3	—	—	90	—	—	280
4	—	—	160	—	—	310
New polycarbonate cage						
1	—	0.14	0.18	—	0.26	0.32
2	—	0.14	NQ	—	0.27	NQ
New polysulfone cage						
1	—	—	NQ	—	—	NQ
2	—	—	0.84	—	—	1.5

Abbreviations: LQ, less than method quantification limit; NQ, not quantifiable because ^{13}C-BPA surrogate not recovered.

of the second sample. However, the level of BPA released from the new polycarbonate cages between the second and third assays was identical, measuring just above the detection limit of the assay (Table 1). In the third replicate, the concentration of BPA in one of the polysulfone cage water samples was greater than that of the new polycarbonate cage but was between 0.5–8.3% of the concentration of BPA detected in the used polycarbonate cage water samples (Table 1). The BPA content of the second new polysulfone cage water sample could not be quantified because of poor recovery of the ^{13}C-BPA surrogate.

No BPA was detected in the water samples from the glass dishes or polypropylene cages (Table 1). However, the GC/MS did detect trace amounts of nonylphenol in the water sample from the second polypropylene cage (data not shown). The glass dish and used polypropylene samples were not run in the second leaching study because the initial leaching study results indicated that no BPA leaching had occurred, and no complementary cell proliferation assay was run on the second leaching study samples.

The two used polycarbonate cages added to the third experiment released even higher amounts of BPA than did the used polycarbonate cages from the first experiment. These two used polycarbonate cages (cages 3 and 4; Table 1) were more pitted and opaque than the first set. The increased wear was likely the cause of the higher amount of BPA leaching

relative to the initial set of used polycarbonate cages. The used polycarbonate cages tested in the first leaching experiment were retested for the release of BPA two more times in the second and third leaching studies. These used polycarbonate cages released lesser amounts of BPA in the second and third experiments relative to the first experiment. The decreased amounts of BPA detected in the replicate experiments were associated with the repeated weeklong extraction periods and repeated rinses before each replicate study (Table 1).

Accompanying the cage water samples were a total of four water blanks and five spiked water quality control samples with varying amounts of BPA (48–318 ng) in the 1-L samples (data not shown). Blank water samples were composed of one sample that was through carbon filtration at the University of Missouri biology building (blank A), one cold tap water sample at the U.S. Geological Survey (USGS) lab (blank B), and two water samples from the USGS lab after combined reverse osmosis/carbon filtration (blanks C and D). The ^{13}C-labeled BPA surrogate standard (labeled 99% pure) was also analyzed for any native BPA background or interference at the same amount (1,000 ng total) added to all samples. In blanks B–D, BPA was not detected (< 7 ng or 0.007 μg/L), and in blank A, BPA was detected at the detection limit (7 ng or 0.007 μg/L) based on ion signal responses exceeding 3× noise. For the surrogate standard, native BPA or background interference was

6 ng, which was approximately the same as blank A. Native BPA-spiked water samples were all determined with the following respective values without background correction. For the highest spike (318 ng), BPA was 330 ng; for the duplicate 159 ng spikes, BPA was 158 and 160 ng; for 80 ng, BPA was 87 ng; and for the lowest (48 ng), BPA was 55 ng. If one accounts for the background in the spiked samples, then background corrected values are almost identical to expected values.

Percent recoveries of the surrogate in all of the quality control samples averaged 86% and ranged from 48% to 115%. Using the isotopically labeled surrogate (isotope-dilution MS technique) with a full range of native BPA calibrations standards, quantitations of native BPA were "self-corrected" by the corresponding degree of recovery of the surrogate. For example, in the duplicate spiked (159 ng) samples, the surrogate recovery varied (48% and 101%), but resulting quantitations of native BPA were virtually identical (158 and 160 ng). Similarly, in the cage water samples, despite some variation in surrogate recoveries, the surrogate technique provided quality assurance for correct native BPA quantitations.

MCF-7 cell proliferation assay. The estrogenic bioactivity of the cage water samples from the third cage leaching experiment was tested in an estrogen-sensitive cell proliferation assay. All water samples from the used polycarbonate cages stimulated cell proliferation (Figure 1). The addition of the antiestrogen keoxifene blocked the increase in cell proliferation, which indicated that the cell proliferation was due to an estrogenic compound acting via estrogen receptors.

Cells treated with water samples from the new polycarbonate cages as well as the new polysulfone cages showed no detectable increase in proliferation; the lack of proliferation with the new cage samples was similar to the results of the untreated control cells. One of the polypropylene cage water samples showed slight estrogenic activity, which can be attributed to the trace amount of nonylphenol detected in the water sample by GC/MS analysis. Importantly, the proliferation response to the leachate from this polypropylene cage was also blocked by addition of antiestrogen. The water samples from the remaining polypropylene cage and the two glass dishes did not stimulate cell proliferation, revealing the absence of bioactive concentrations of estrogenic compounds in these samples.

HPLC analysis. The HPLC analysis of water sample from used polycarbonate cage 4, which contained substantial estrogenic activity in the MCF-7 cell proliferation assay, showed that the estrogenic activity was associated with a single peak that coeluted with BPA (Figure 2A). There was no significant estrogenic activity coeluting with natural estrogens (estrone,
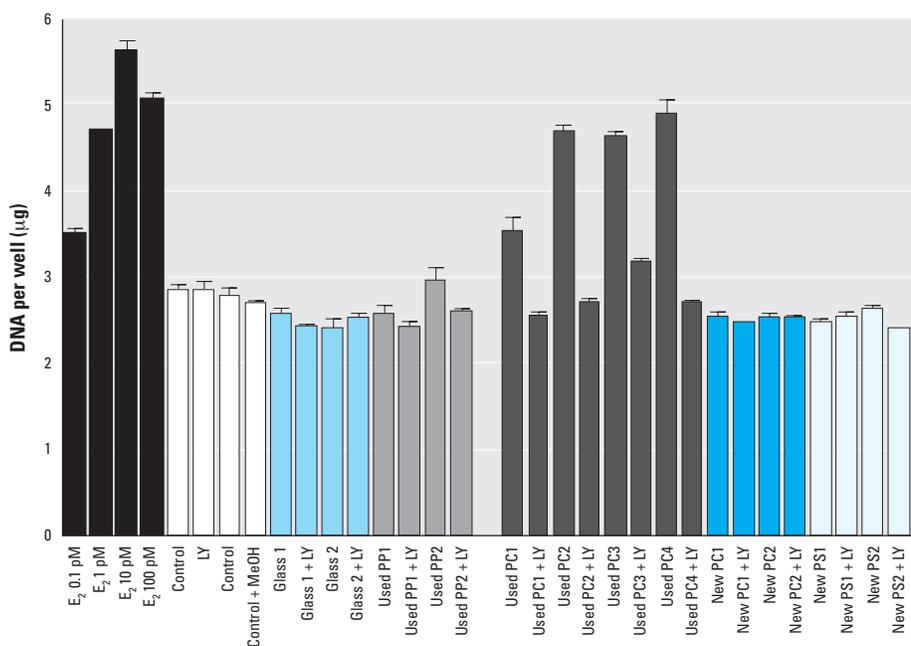
Figure 1. Cell proliferation in MCF-7 cells exposed to water samples of plastic animal cages. Abbreviations: Control, control estrogen-free media; Glass, glass dish; LY, an antiestrogen, also called keoxifene; MeOH, methanol; PC, polycarbonate cages; PP, polypropylene cages; PS, polysulfone cages. Cells grown in phenol red–free (estrogen-free) maintenance medium were treated for 4 days with the media containing the reconstituted cage and glass dish water samples, a range of 17β-estradiol (E$_2$; 0.1–100 pM), and/or 0.1 mM LY156758 with daily medium changes. Values are the mean ± SE; $n = 3$.

estradiol, or estriol), conjugates (e.g., estrone-3-sulfate), phytoestrogens (e.g., genistein), or possible synthetic contaminants such as diethylstilbestrol (DES) (Figure 2A). A negative control polypropylene cage extract showed no peaks of significant estrogenic activity near BPA or any other estrogens in the series (Figure 2B). These findings are thus consistent with the GC/MS results that the cell-proliferative

response was caused by the BPA content of the used polycarbonate cage water samples, rather than any estrogens from mouse urine remaining in the cage walls after the sanitizing cage wash and rinses before the experiment, or any estrogen contamination in the laboratory.

Prepubertal mouse uterotropic assay. To study the *in vivo* effects of BPA exposure through cage and water bottle materials, we

measured the uterine wet weight of prepubertal female mice housed in used polycarbonate cages with used polycarbonate water bottles versus mice housed in used polypropylene cages with glass water bottles; the objective was to establish the two extreme conditions of maximum and lowest exposure to BPA via caging materials. On postnatal day 19, there was no difference in body weight at weaning for females placed in used polycarbonate cages versus females placed in used polypropylene cages. The plastic cage type did not influence the body weight of female mice at time of collection on postnatal day 26. There was a significant ($p < 0.001$) relationship between uterine wet weight and body weight, so the uterine wet weight data were adjusted for body weight at collection by ANCOVA. The data were also adjusted for litter effects because more than one individual per litter was used. There was no significant difference in uterine wet weight based on housing in used polycarbonate versus used polypropylene cages (Table 2).

Discussion

Our results support the findings of Takao et al. (1999), who reported an increased rate of leaching from polycarbonate plastic with age. Our findings were similar to those of Takao et al. (1999) in that we detected higher levels of BPA migrating from the used polycarbonate cages than from the new polycarbonate or new polysulfone cages. A difference between our study and prior studies of the leaching of BPA is that we examined whether BPA would migrate passively out of used polycarbonate caging under the moderate conditions of standing undisturbed (not shaken during the experiment), normal room temperature, and a neutral, polar solvent (HPLC-grade water).

Polycarbonate and polysulfone are both strong thermoplastics that are, in most cases, made using BPA. Technically called copolymers, they are produced by reacting two different molecules together, resulting in combined copolymer units ($n = 50–100$ units; Figure 3) with a molecular mass of approximately 25,000 Da per individual copolymer. BPA is a symmetrical aromatic molecule that reacts on both phenolic ends in polymerization reactions. For polycarbonate, BPA typically reacts with phosgene, forming an ester linkage, whereas for polysulfone, BPA typically reacts with dichlorodiphenyl sulfone to form an ether linkage. The copolymers' strength comes from the rigid aromatic rings, and inherent flexibility comes from the ether and ester C—O single bonds, which are freer to rotate, as demonstrated in the nonlinear drawing of Figure 3. Both copolymers are amorphous (i.e., they do not form a crystalline structure) but can be melted and formed or reformed into strong structures and thus are suitable for use as thermoplastics.
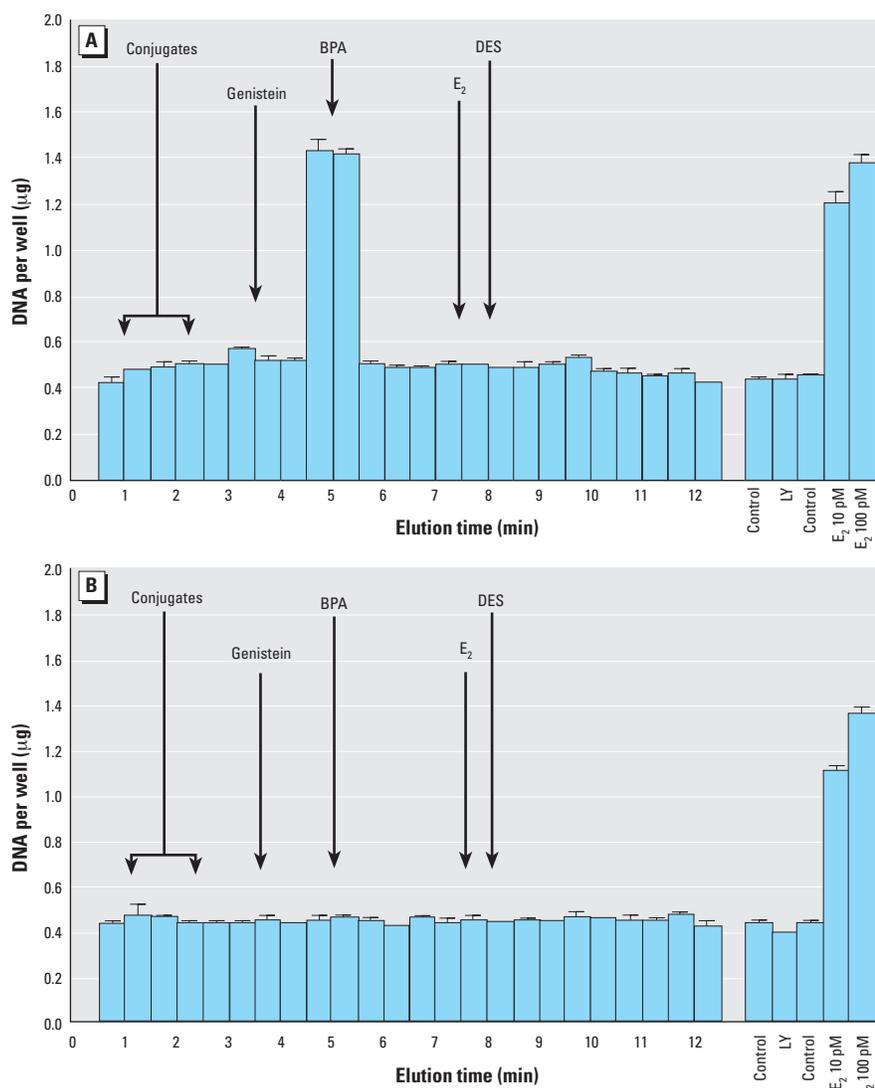
Figure 2. HPLC/bioassay separations of a water extract of a used polycarbonate cage (*A*) and a polypropylene cage (*B*), separated in 60:40 methanol:water as described in Grady et al. (1991). Abbreviations: Control, control estrogen-free medium; E_2, estradiol; LY, 100 nM antiestrogen LY156758. Fractions are shown from 0.5 through 12.5 min. Arrows indicate approximate elution times of standards, separated in separate HPLC runs. Estrogenic activity as increased proliferation is shown on the vertical axis. The bars to the right of the separation are controls. Values are the mean ± SE; $n = 3$.

Table 2. Prepubertal mouse uterotrophic assay assessing estrogenic influence of used polycarbonate animal cages versus polypropylene cages.

Housing condition	PND19 bw (g)	PND26 bw (g)	Uterine wt (mg)
PP cages with glass bottles ($n = 57$)	9.75 ± 0.16	16.30 ± 0.2	17.25 ± 0.70
Used PC cages with used PC bottles ($n = 57$)	10.11 ± 0.16	17.12 ± 0.24	20.56 ± 1.13
Statistical significance	$p = 0.17$	$p = 0.83$	$p = 0.31$

Abbreviations: bw, body weight; PC, polycarbonate; PND, postnatal day; PP, polypropylene; wt, weight. All data shown are mean ± SE; n = number of mice per housing condition.

Polymerization reactions may approach 100% but rarely achieve it (Cowie 1991). Usually some amount of reactants remain in the finished product.

Factor (1996) noted that "perhaps the most important but most easily overlooked aspect of BPA-polycarbonate stability is its vulnerability to reaction with water . . . both during melt processing and in end-use applications involving exposure to water at elevated temperatures, such as sterilization by autoclaving." Industry minimizes the susceptibility by removing catalytic residues, carefully drying before melt processing, and adding certain stabilizers (Factor 1996). However, polycarbonate as an ester is susceptible to hydrolysis and base-catalyzed hydrolysis, mainly at elevated temperatures, while remaining resistant to hydrolysis at ambient temperatures (Thompson and Klemchuk 1996). However, although our findings confirm that this applies to new polycarbonate cages, our findings show that as polycarbonate cages age, associated with discoloration and cracking, there is a marked increase in leaching of free BPA into water at room temperature.

Although the solubility of water (as liquid) in the copolymer is very low, some water (as vapor) dissolves in it when immersed in boiling water. Subsequently, when cooled, the copolymer appears hazy because small water droplets have formed and condensed within the matrix. Ram et al. (1985) found no mechanical defects from immersing polycarbonate in water at room temperature for 1 year but 16% shrinkage after 30 days at 40°C, 55% after 30 days at 60°C, and total "tensile breakdown" after just 14 days at 80°C. Even the most stable grade of polycarbonate failed in hot water, although not as quickly. Thus, BPA is released as a hydrolysis breakdown product of the repeated washing in the sanitizing cage washer (Factor 1996) and as unreacted molecules via the increased surface area of the cage interior as a result of wear. The polysulfone copolymer is predicted to be less susceptible to hydrolysis due to the ether bond between polysulfone copolymers, relative to the ester bond in polycarbonate copolymers; however, our laboratory has not tested the durability or the amount of BPA leaching from used polysulfone cages relative to polycarbonate cages.

We found a decrease in the amount of BPA that was released from the used polycarbonate cages when examined multiple times in three leaching experiments. This was probably due to the fact that the first leaching experiment was run on cages that had been recently cleaned in the industrial sanitizing cage washer and then stored, thus allowing for the accumulation of free BPA on the surface of the cages. The rinses in tap water and HPLC-grade water were intended to remove the free BPA and other residues on the surface of the cages before the beginning of each replicate of the leaching experiment. Thus, the initial amount of free BPA available to leach into the water should have been reduced in the second and third replicates relative to the first experiment with the used polycarbonate cages.

The results of the second and third replicates of the leaching experiment thus revealed the amount of BPA that is steadily released into room temperature water by the used polycarbonate cages over a 1-week period, after at least some of the free BPA had been removed from the surface during preexperiment rinsing and previous week-long replicate leaching experiment(s). However, the results from the first leaching experiment are likely to be the most representative of the actual exposure of animals housed in polycarbonate cages that were washed in a sanitizing cage washer and then stored before use. Indeed, because we rinsed the cages before the start of each of the three replicate leaching experiments, the amount of BPA recovered from the cages during the experiment is likely to be less than what would have been found without a series of rinses before the start of each replicate experiment.

The difficulty with measuring BPA at levels near the detection limit in water from the new polycarbonate and polysulfone cages in the final experiment likely resulted from the very polar nature of BPA. The two phenolic groups of BPA provide sites for hydrogen bonding from any active sites on the film of the capillary column, which can produce peak tailing or broadening of the peak, with a significant drop in peak height giving a much lower signal-to-noise value. The presence of the coeluting ^{13}C-BPA surrogate aided the chromatographic process of separating and measuring BPA in each sample, along with assuring accurate quantitation by GC/MS. However, because of significant tailing, especially when no or minimal levels of native BPA are in a sample, it is sometimes difficult to accurately measure the percent recovery of ^{13}C-BPA using the nonpolar internal standard D_{14}-p-terphenyl. This explains why, in two cases, we could not quantify BPA in a sample (Table 1).

The sensitivity of the MCF-7 cell line to endogenous estradiol and estrogen-mimicking BPA has been well characterized (Nagel et al. 1997; Samuelsen et al. 2001; Villalobos et al. 1995; Welshons et al. 1999). In particular, MCF-7 cells have been used to detect the estrogenic activity of BPA-containing products, such as the epoxy resin lining in food cans (Brotons et al. 1995) and some dental sealants (Olea et al. 1996). In the present study, BPA migrating from the used polycarbonate cages stimulated a significant increase in MCF-7 cell proliferation compared with control media and glass dish negative controls.
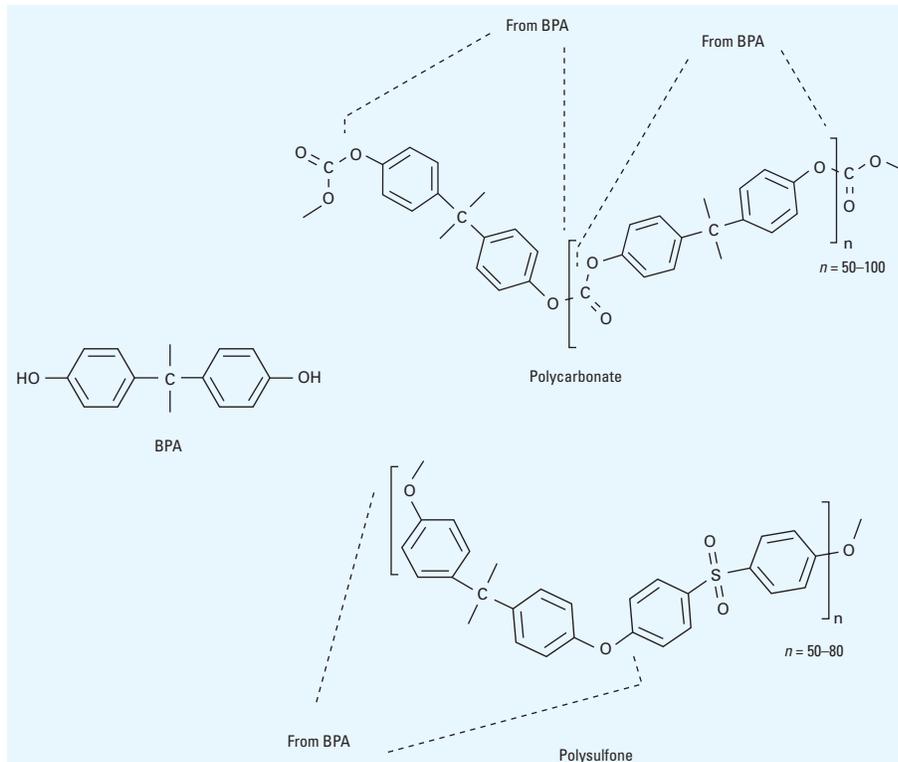
Figure 3. Structure of BPA and partial structure of the copolymers polycarbonate and polysulfone shown by monomeric chain units (n) within brackets. Both the rigidity of the aromatic rings and the inherent flexibility of the C—O, C—S, and C—C single bonds are depicted. Polycarbonate is joined by ester linkages (O—C=O—O) whereas polysulfone has ether linkages (C—O). For images of three-dimensional structures, refer to Edge et al. (1994).

That the bioactivity of the leachate from used polycarbonate cages was mediated via estrogen receptors was confirmed by the subsequent addition of an antiestrogen, which inhibited the estrogen-mediated proliferative effect. A trace amount of nonylphenol, another estrogen-mimicking chemical, was detected in the water sample from a polypropylene cage by the GC/MS analysis and was likely responsible for stimulating a detectable increase in cell proliferation. The nonylphenol likely migrated from the polypropylene plastic material; however, it also could have been leftover detergent residue on the cage wall.

Prepubertal uterine wet weight was approximately 16% greater in females housed in the used polycarbonate caging with used polycarbonate water bottles relative to mice housed in used polypropylene cages, although the results were not statistically significant ($p = 0.31$). These data suggest that the level of BPA exposure from used polycarbonate cages and bottles was not sufficient to elicit a uterine wet-weight response relative to that which occurs when estradiol is administered to prepubertal female mice (Shelby et al. 1996). However, our subsequent findings and other recent published reports indicate that uterine wet weight gain in female mice is not a sensitive bioassay for BPA estrogenic activity. Specifically, Markey et al. (2001b) reported that a 100 mg/kg/day dose of BPA was required to induce significant increases in uterine weight in prepubertal CD-1 female mice, whereas fetal exposure to a 4,000-fold lower dose (25 µg/kg/day) of BPA (via the mother) stimulated the mammary ducts when the female offspring were examined in adulthood (Markey et al. 2001a). Nagel et al. (2001) also reported that BPA stimulated significant uterine wet weight gain only at a high dose of 25 mg/kg/day in adult, ovariectomized estrogen-receptor indicator (ERIN) mice, which are engineered with a β-galactosidase reporter gene attached to the mouse estrogen receptor. However, BPA stimulated transcriptional activity of the estrogen receptor at doses 1,000-fold lower than those stimulating uterine wet weight gain.

In contrast to the insensitivity of the uterine wet-weight response, the fetal male prostate was significantly ($p < 0.05$) enlarged by either a low dose of BPA (50 µg/kg/day) or a low dose of DES (a potent synthetic estrogen; 0.1 µg/kg/day) administered to pregnant CD-1 mice relative to vehicle-fed controls (Gupta 2000). Taken together, these findings reveal that relative to other bioassays, uterine weight gain in female mice is a very insensitive bioassay for estrogenic activity of BPA. The much higher estrogenic potency of BPA in other tissues suggests that BPA acts as a selective estrogen receptor modulator and exhibits unique responses relative to estradiol in different target tissues.

Previous studies have shown that exposure to low, environmentally relevant levels of BPA have a significant effect on reproductive function in rodents. For example, prenatal exposure to low doses of BPA resulted in accelerated growth and timing of puberty (Honma et al. 2002; Howdeshell et al. 1999), altered estrogen receptor expression patterns in the vagina (Schönfelder et al. 2002a), and increased proliferation of mammary tissue (Markey et al. 2001a). In male rodents, developmental exposure to BPA increased prostate weight (Elswick et al. 2000; Gupta 2000; Nagel et al. 1997), decreased epididymal weight (Gupta 2000; vom Saal et al. 1998), and decreased daily sperm production (Sakaue et al. 2001; vom Saal et al. 1998). Still other studies have reported no significant effects of low-dose BPA exposure (Ashby et al. 1999; Cagen et al. 1999); however, neither of these studies was able to demonstrate an effect with their positive control that makes the results of other experimental groups questionable. It remains to be determined whether any of these effects observed in response to low doses of BPA will be observed in animals housed in old polycarbonate cages and with old water bottles.

Our findings here suggest that aquatic laboratory animals may be exposed to BPA due to leaching from worn polycarbonate caging in sufficient amounts to significantly affect reproductive parameters. This prediction is based on a number of recent reports of significant effects at very low concentrations of BPA in frogs, fish, and mollusks. In the South African clawed frog (*Xenopus laevis*), exposure of tadpoles to a low dose of BPA (22.8 µg/L or 10^{-7} M) in water for 12 weeks changed the sex ratio by increasing the number of females relative to controls, similar to a 2.8 µg/L (10^{-8} M) dose of estradiol (Kloas et al. 1999). In guppies (*Poecilia reticulata*), BPA exposure (274 µg/L for 21 days) in adulthood significantly decreased the number of mature sperm stored in deferent testes canals before ejaculation by 50%, relative to control males (Haubruge et al. 2000). A significant decrease in spermatozoa has also been reported for fathead minnows (*Pimephales promelas*) after exposure to 16 µg/L BPA in water for 164 days in adulthood (Sohoni et al. 2001). In the Japanese medaka (*Oryzias latipes*), exposure to 10 µg/L BPA in water during the first 100 days of life resulted in the presence of ovo-testes, which was noted in a few males; exposure to 50–200 µg/L BPA produced testicular abnormalities, including a decrease in the number of spermatozoa (Metcalfe et al. 2001). Also in the medaka, the induction of female specific proteins was reported in adult males exposed to 10 ppb (10 µg/L) BPA in water for 5 weeks (Tabata et al. 2001). Finally, Oehlmann et al. (2000) reported reproductive organ abnormalities and

abnormal oocyte production in freshwater and marine snails (*Marisa cornuarietis* and *Poecilia reticulata*) exposed in adulthood to 1 µg/L BPA (the lowest dose tested).

In summary, these findings suggest that further studies are needed to investigate whether leaching from used polycarbonate cages or other BPA-containing plastic animal housing materials, such as polycarbonate or polysulfone cages and bottles, may influence the physiology of the animals and/or their responsiveness to experimental treatments. There are other sources of BPA exposure in the laboratory. BPA migration into human serum has been reported with the use of polycarbonate and polysulfone plastic hemodialysis equipment (Yamasaki et al. 2001). Another potential route of BPA exposure in the laboratory is polyvinyl chloride (PVC) pipes used in the supply of tap water; BPA is added as a stabilizer in the production of PVC products. Carbon filters are effective in removing phenols, such as BPA, from water, and our animal colony water is purified by ion exchange followed by a series of carbon filters. We have also always used polypropylene cages and glass water bottles in our experiments with mice. Researchers should be aware of and control for possible sources of estrogenic chemical contamination of their laboratory animals. In particular, researchers housing aquatic research animals in polycarbonate cages need to be aware of the potential for significant exposure to free BPA as the cages age.

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