Maisons-Alfort, 31 May 2010

ANNEX

to AFSSA's Opinion dated 29 January 2010 on the critical analysis of the results of a developmental neurotoxicity study of bisphenol A together with other recently-published data on its toxic effects

This annex summarises the methodology and expert assessment of the documents examined in AFSSA's Opinion dated 29 January 2010 on the critical analysis of the results of a developmental neurotoxicity study of bisphenol A together with other recently-published data on its toxic effects.

1. METHODOLOGY

The articles and scientific reports reviewed by the "Bisphenol A" Working Group (BPA WG) and listed in the references came from an active literature search undertaken at AFSSA's Department for the evaluation of nutritional and health risks between June 2009 and January 2010, using the SCOPUS and PUBMED databases and searching for "bisphenol A" in the title / abstract / body text. Few references predating 2009 were selected from the references cited in these articles and from the searches made by the BPA WG.

This Annex focuses on the analysis of the effects of BPA in laboratory animals (rodents, primates), and in particular the effects observed after exposure to levels below the no observed adverse effect level (NOAEL) of 5 mg/kg b.w./day on which is based the tolerable daily intake (TDI) of 0.05 mg/kg b.w./day established by EFSA[1] in 2006. Studies primarily selected were those in which administration was by oral (gavage) or dietary (inclusion in feed or drinking water) route.

The critical review of each study's experimental method particularly took into account factors that could alter or mask the action of BPA at low doses:

- composition of the diet: the presence of phytoestrogens,
- composition of the animals' housing cages and drinking water containers: the presence of polycarbonate,
- composition of the drinking water: tap water, treated water (activated carbon filtration), BPA concentration,
- composition of the bedding: presence of endocrine disruptors.

The literature reviews compiled by the *Réseau Environnement Santé* network (a French non-governmental organization) and sent to AFSSA in July, October and November 2009 were also taken into account. Furthermore, the *Réseau Environnement Santé* was invited to present a literature review at a hearing held on 1 December 2009.

Each of the selected scientific papers was analysed by at least two members of the working group and was discussed in the course of the three meetings. The findings of the BPA WG, which served as the basis for AFSSA's Opinion of 29 January 2010, were presented and discussed at plenary meetings of the "Food Contact Materials" and "Physical and Chemical Contaminants and Residues" Scientific Panels, which were held on 21 and 13 January 2010 respectively.

This analysis excluded studies involving non-oral routes of administration, such as subcutaneous or intraperitoneal injections, or via pumps under the skin, except when these yielded comparable parameters to the results of oral studies. Similarly, studies whose assessment criteria were only intended for mechanistic understanding (e.g. modulation of expression of certain genes) were not included.

[1] European Food Safety Authority

The methodological characteristics (principally the number of animals per group, the number of doses tested, the presence of a positive control) and the main results obtained in each study are summarised and discussed. Study quality was assessed according to the approach of Klimisch *et al.* (1997), as described by AFSSET in a guidance document in July 2007[2]. Rating ranged from 1 to 4, according to the criteria shown in Table 1. The references selected for analysis are listed by subject and in alphabetical order.

Table 1: Rating criteria according to the approach of Klimisch *et al.* (1997)

RATING	CATEGORY OF VALIDITY
1 - 1a - 1b - 1c - 1d	**Valid without restriction** - GLP study respecting standardised tests (OECD, EC, EPA, FDA, etc.) - Comparable to a standardised study - Protocol in accordance with a national standard method (AFNOR, DIN, etc.) - Protocol in accordance with scientifically accepted standard methods and described in sufficient detail
2 - 2a - 2b - 2c - 2d - 2e - 2f - 2g	**Valid with restrictions** - Standardised study without detailed documentation - Standardised study with acceptable restrictions - Comparable to a standardised study with acceptable restrictions - Protocol in accordance with national standard methods, with acceptable restrictions - Well-documented study, consistent with scientific principles, acceptable for assessment - Accepted method of calculation - Data from reference works and data collection
3 - 3a - 3b - 3c	**Invalid** - Documentation insufficient for assessment - Significant methodological deficiencies - Unsuitable protocol
4 - 4a - 4b - 4c - 4d - 4e	**Not assignable** - Abstract - Secondary literature - Original reference not available - Original reference in a foreign language (not English) - Documentation insufficient for assessment

For studies relating to the release of BPA from polycarbonate baby bottles and other containers (food and beverage cans), Klimisch's rating system could not be applied. The following classification was therefore adopted:

1 = new data,
2 = confirmation of data already published,
3 = not relevant in the context of the expert assessment undertaken by the BPA WG.

[2] http://www.afsset.fr/upload/bibliotheque/665904925967833733338285157123/07_32_ccap.pdf, only available in French

2. IDENTIFICATION OF EXPERIMENTAL BIASES

Several parameters were identified as potential sources of experimental bias in toxicity studies of BPA in laboratory animals (apart from the number of animals tested per group, the number of doses, the presence of positive and negative controls):

- nature of the housing cages / drinking water containers for the animals,
- composition of the diet,
- method of administration,
- water quality,
- nature of the bedding.

2.1 Nature of the cages

The study by Howdeshell *et al.* (2003) emphasised the importance of the nature of the plastic used in the manufacture of rodent housing cages. The authors studied BPA migration from the walls of cages made of polycarbonate (new and used), polysulfone, polypropylene and glass. To do this, water at a neutral pH was put in cages placed at room temperature. After one week, the water was recovered and its possible estrogenic effect was determined by a proliferation assay on MCF7 cells (E-Screen). The effect of the cages was also investigated *in vivo* on immature mice by a uterotrophic assay.
The results show that only polypropylene and glass cages did not release BPA. Polycarbonate and polysulfonate cages released a small amount when new (respectively 0.3 and 1.5 µg/L), whereas used polycarbonate cages released 1000 times more than new ones (310 µg/L compared with 0.3 µg/L). Prepubertal female mice placed in used polycarbonate cages had a mean uterine weight (calculated from more than 20 litters of at least six 19-day females) 16% higher than females placed in polypropylene cages. The authors concluded that the animals were subjected to chronic exposure to bisphenol A, through contact or through licking of the walls.

2.2 Composition of the diet

Most commercial rodent diets use soy as a source of protein and therefore contain phytoestrogens such as isoflavones and coumestans whose estrogenic properties are well documented. However, the literature also refers to other diets which, in general, can be classified into 3 types:

- totally semi-synthetic diets in which the protein intake is in the form of casein (purified diets, rarely used),
- conventional pellet form diets in which the protein intake comes from soybeans or other pulses: in these diets, phytoestrogen levels vary, and mostly do not exceed 600 mg/kg. However, comparative studies make it possible to observe the impact of the presence of phytoestrogens in diets on metabolic and behavioural studies.
- diets specifically developed for endocrine disruption studies, which lack soy protein and thus phytoestrogens. Several articles indicate that the presence of phytoestrogens in the diet has an impact on the estrogen response.

In 2003, *Owens* et *al.* published the results of a study on phytoestrogen levels in commercial diets used in the OECD programme to validate the rat uterotrophic bioassay. These validation tests were conducted with BPA and nonylphenol on immature females and ovariectomised adults. Phytoestrogen concentrations (the sum of genistein, daidzein and coumestrol levels) in the commercial diets ranged from 150 to 350 mg/kg diet, but some could contain up to 600 mg/kg. The results showed that the relative uterine weights (with reference to the full body weight) were correlated with the phytoestrogen content in the diets. A uterotrophic effect was observed for diets containing high levels of isoflavones, whereas low or moderate levels did not seem to affect the uterotrophic test's response to nonylphenol or BPA (tested at a dose of 600 mg/kg/d).The authors concluded that the use of diets containing levels of phytoestrogens below 325-375 mg/kg does not interfere with the result of the uterotrophic test. While this study provided precise data on phytoestrogen levels in the diet that may influence the uterotrophic effect of BPA in female rats, measured in accordance with the OECD guidelines, it used a high dose of BPA and only considered the weight of the uterus.

In a literature review, Jensen *et al.* (2007) identified most of the commercial diets used in animal experiments and analysed the studies in order to describe the phytoestrogen levels of these diets and the observed effects on different targets (uterotrophic test, bone mineralisation, carcinogenesis, etc.). This review confirmed that genistein and daidzein are the main phytoestrogens found in soy-based diets. The authors concluded that diets containing high concentrations of phytoestrogens affect the experimental response. They also concluded that not all biological targets have the same sensitivity and urged caution in interpreting results obtained in the presence of phytoestrogens. While in many studies the thresholds above which responses are influenced by phytoestrogens are between 300 and 400 mg/kg food, some studies have shown that some toxicological targets, such as the behaviour or development of hormone-dependent cancers, may be affected by much lower levels.
This analysis had the advantage of taking into account several types of protocol including perinatal exposure and long-term studies. It cautioned with regard to the multiple effects that phytoestrogens may have in endocrine disruptor studies. It is unfortunate that this analysis did not include any totally soy-free diets.

Thigpen *et al*., (2007) evaluated the effect (growth, vaginal opening, anogenital distance) on rodents of diets with different concentrations of phytoestrogens. In this study, the authors fed CD1 mice and F344 and Sprague Dawley rats, from gestation, with commercial diets whose phytoestrogen content was measured. The results showed that for a given species and strain, the daily weight gain, anogenital distance and vaginal opening were correlated with the level of phytoestrogens in the diet. For a given diet, the effect on the progress of puberty varied from one species to another and the authors identified the Sprague Dawley rats as weakly sensitive and therefore not the most suitable model for endocrine disruption studies. For diets with a very low phytoestrogen content, vaginal opening may be very late (42nd day).
This study showed the importance of diet, and particularly feed phytoestrogen levels, in the estrogen response, and suggested avoiding the use of commercial diets with high levels of phytoestrogens for the detection of such effects.

The work of Ruhlen *et al*. (2008) in CD1 mice aimed to compare commercial diets with or without soy, on criteria of toxicity to reproduction and development. The experimental design consisted in feeding mothers and the corresponding F1 generation with a soy-free diet (PMI 5K96) in which protein intake was mainly provided by casein. In parallel, under the same conditions, animals were fed with soy-based diets (PMI 5008 [gestation/lactation] and PMI 5001 [post-weaning]). The phytoestrogen levels of the diets were not measured, but the estrogenic activities of methanol extracts from each feed were evaluated by a cell proliferation assay (MCF7).
The results, expressed in mg/kg of genistein equivalent, were 3.9 for the PMI 5K96 feed, 40.0 mg/kg for PMI 5008 and 25.8 mg/kg for PMI 5001. The main parameters measured were circulating levels of estradiol during gestation in mothers and foetuses, the weight of newborn and young, the weight of various organs related to reproduction, adipose tissue in the F1 generation and circulating levels of leptin. Contrary to expectations, serum estradiol levels were higher in foetuses whose mothers were fed the soy-free diet than in foetuses from the group receiving the conventional diet, and these levels were associated with adverse effects on reproduction indicative of foetal estrogenization syndrome. In females, it induced precocious puberty and increased the uterotrophic response to estradiol. In males, it increased prostate volume and caused an alteration in reproductive parameters as with estrogenic compounds. In adulthood, these animals became obese and produced abnormal levels of leptin.
The authors concluded that laboratory animals fed with diets rich in phytoestrogens for several generations develop an adaptive process which results in an estrogenization syndrome when they change to a diet free from phytoestrogens, which has implications for experimental studies designed to identify endocrine disruptions. While this warning should be taken into account, data on the metabolic effects should be interpreted with caution due to substantial differences in the composition of diets (levels of lipids, proteins, carbohydrates, proportion of sucrose).

Nevertheless, other studies have reported metabolic disturbances induced by phytoestrogens. In 2004, Lephart *et al.* compared in rats the effects on food intake, feeding behaviour, body fat and serum parameters related to general metabolism, of two diets, one rich (600 mg/kg) and the other poor (15 mg/kg) in soy isoflavones. The study began from conception and continued until adulthood. It showed that animals fed with a diet without soy were fatter and had higher leptin levels while eating less than the others. These effects were accompanied by increased circulating levels of insulin, decreased levels of glucose and T3, and less effective temperature control, leading the authors to conclude that the phytoestrogens in the diet affected general metabolism and feeding behaviour.

This study highlights the pro-adipogenic effects of soy-free diets but does not exclude a nutritional effect due to other constituents, because of a lack of precise information on the composition of the soy-free diet and on the origin of the proteins in this diet.

Hartley et *al.* (2003) studied the impact of phytoestrogens in the diet on the social behaviour of adult male rats. This work focused on two groups of 60 rats, one fed a soy-free diet (Harlan Teklad 2016) and the other fed a diet containing 150-250 mg of isoflavones (genistein and daidzein) per kg feed (Harlan Teklad 2018). Consumption of food and water were measured for 14 days, then the animals were placed in individual cages for 4 days before performing behavioural tests (social interaction test and measurement of anxiety). Corticosterone levels were measured before and after stress induced by the two anxiety tests. Vasopressin and oxytocin were assayed immediately after the induction of manual stress. No effects were observed on food consumption, growth or the level of oxytocin. Baseline levels of cortisol and vasopressin were identical in both groups, but animals receiving the diet rich in phytoestrogens had higher rates after the induction of stress. In the group receiving the diet containing soy, the animals were less active and more anxious.
These results indicate that the presence of phytoestrogens in the diet can influence the behaviour of animals and create an experimental bias. As this study was performed on adults, the neurobehavioural effects observed were not linked with any effect on development. Moreover, there is every reason to believe that these effects are rapidly reversible. Indeed, before beginning the experiment, all animals were subjected to standard diets containing 150 mg/g of soy isoflavones (genistein and daidzein), which did not prevent a difference being observed between the two groups after a trial period of only 14 days.

These data show that the presence of phytoestrogens affects the estrogenic response (especially on the uterotrophic test), general metabolism, development, and social and feeding behaviour.

2.3 Method of administration

Most studies involving low doses rely on administration by subcutaneous injection, which controls more precisely the amount administered per day and per unit of body weight. The use of osmotic pumps facilitates studies of chronic exposure, but these administration methods have the drawback of not taking into account toxicokinetic parameters such as bioavailability, or intestinal or liver metabolism.
Studies relating to food contamination tend to prefer oral exposure, either using gavage probes or depositing the test compounds directly in the oral cavity using a micropipette (Palanza *et al.).* In this case, the vehicle used to solubilise and administer the test substances may affect absorption or introduce compounds which might themselves be active on the studied targets. Thus, protocols using olive oil, which is rich in polyphenols, mean that the possible risk of interaction between these polyphenols and endocrine disruptors tested at low doses cannot be ruled out.

In order to estimate the impact of the administration route on forms and circulating levels of BPA, Taylor *et al.* (2008) administered single doses of radiolabelled BPA to mice. A first group received a subcutaneous injection of BPA and a second was treated by gavage. Two doses were tested (35 and 395 µg/kg) and plasma concentrations were measured in the 24 h after treatment. Although the kinetics at 3 and 6 h differed significantly between the two groups, plasma levels of free BPA were similar at 24 h, suggesting that at the doses tested, the route of administration did not affect circulating levels. Nevertheless, this study did not take into account conditions of repeated administration, which are closer to the reality of exposure to BPA.

2.4 Water quality

Tap water may contain chemical contaminants at trace levels, some of which exhibit endocrine disruption activity. BPA may be used to manufacture materials used in drinking water systems. The possible presence of BPA in tap water should therefore be considered in the context of animal experiments. Filtration on granular activated carbon has highly variable efficacy, with elimination percentages ranging from 25-75% (Stackelberg *et al*., 2007).

Note that, in France, the Midi-Pyrenees DRASS (Regional health and social affairs authority) and the Adour Garonne Water Agency conducted an analysis campaign in 2006-2007. BPA was screened for

in 10 samples of raw groundwater, 31 samples of raw surface water and 40 samples of drinking water intended for human consumption. All results were below the method's limit of quantification of 50 ng/L.

All the data from the literature mention concentrations of around a nanogram per litre for BPA potentially present in drinking water intended for human consumption.
Thus, Wenzel *et al.* (2003) detected BPA in surface waters (rivers) in Austria, Belgium, Switzerland, Germany and the Netherlands, at concentrations of tens or hundreds of ng/L (0.8 µg/L maximum). In drinking water, the results were below the detection limit of 8 or 11 ng/L (one water supplier reported a value of 0.12 µg/L which was higher than the value in the corresponding raw water).
The work of Kuch & Ballschmiter (2001) in Germany revealed BPA concentrations ranging from 500 pg/L to 16 ng/L in river water and 300 pg/L to 2 ng/L in drinking water.

2.5 Nature and quality of the bedding

Bedding can be made from sawdust, wood chips or sometimes corn cobs. Some wood (coniferous, eucalyptus) contains potentially estrogenic compounds (polyphenols, terpenes) which may be absorbed by the animals (through licking). Similarly, corn cobs may be contaminated by zearalenone, a mycotoxin that is more estrogenic than soy phytoestrogens and known to be responsible for infertility in some livestock farms.
To date, very few studies have specified how this bedding was taken into account in the experiments.

3. DETAILED ANALYSIS OF BPA TOXICITY STUDIES

The tolerable daily intake (TDI) of BPA of 0.05 mg/kg b.w./d was established by EFSA in 2006 from a no observed adverse effect level of 5 mg/kg b.w./d in a two-generation reproductive toxicity study in mice (Tyl et *al.,* 2006) and a safety factor of 100 (10 for inter-species variability and 10 for inter-individual variability).

Toxicity studies reviewed by the BPA WG were classified into three categories according to the results observed at doses below 5 mg/kg b.w./day:

1. studies finding no toxicity,
2. studies considered as warning signals,
3. studies whose results were not considered by the BPA WG to be of concern.

3.1 Studies finding no toxicity at doses below 5 mg/kg b.w./d

3.1.1 A dietary developmental neurotoxicity study of bisphenol A in rats, WIL-186056, September 2009, 4796p.

Stump *et al*. (2009), American Chemistry Council

Rating 1a

Study objective:
To examine the neurotoxic effects (morphological and/or functional) in young rats after continuous exposure of the mother during gestation (embryonic and foetal development) and during lactation until weaning (21 days after birth, postnatal development): the study was conducted according to the OECD Guideline 426 exploring principally motor functions, learning and memory, as well as changes to brain morphometry and neuropathology.
Method:
- Animals: Crl:CD(SD) rats, 24 females/dose
- Administration:
    - doses of 0.15, 1.5, 75, 750 and 2250 mg/kg feed (corresponding to 0.01, 0.12, 5.85, 56.4, 164 mg/kg b.w./day during gestation and 0.03, 0.25, 13.1, 129, 410 mg/kg b.w./day during lactation, given the average feed consumption).
    - supplementary study: 2250 mg/kg feed.
    - duration of exposure of the main study: GD0 to PND21 (first gestational day until weaning, on the 21st postnatal day)
    - duration of exposure of the supplementary study: GD0 to PND11 (first gestational day until the 11th postnatal day)
- Parameters studied:
    - mothers (F0): body weight, food consumption, detailed clinical examination, weight and microscopic examination of liver and kidney.
    - offspring (F1): daily observation, weighed and physical examined at regular intervals, righting reflex, *in situ* brain perfusion, neuropathology and morphometry, motor activity, learning and memory (Biel water maze), auditory startle, balanopreputial separation, vaginal opening.

Results:
According to OECD guideline 426, the highest dose is determined in order to induce some maternal toxicity (e.g. reduced weight gain of 10% compared to controls). This effect was observed at doses of 750 and 2250 mg/kg feed as a reduction in weight gain of 9.5% and 22.4% respectively, but only during gestation, accompanied by a parallel reduction in food consumption. No other signs of maternal toxicity were noted by the authors. Hair loss observed in dams at 750 and 2250 mg/kg feed and slight agitation of the treated animals were not considered to be related to BPA.
Even in the presence of maternal toxicity at the two highest doses, no deleterious effect on physical and behavioural development of the F1 generation was noted by the authors.
In view of the clonic "popcorn" convulsions observed in some F1 individuals at the higher doses of the range tested (one male and one female at 750 mg/kg feed from 22 individuals of each sex, 1 male and 3 females at 2250 mg/kg feed from 23 individuals of each sex), an additional study was conducted on a larger number of litters in order to reproduce the main study, but only at the highest dose and up to the 11th day after birth (PND11). No convulsions were then observed. An analysis of historical data shows that this type of convulsion at PND11 has been observed previously, but at a lower frequency and only in females (2/244).

Authors' conclusions from the study:
The no observed adverse effect level (NOAEL) identified by the authors was 75 mg/kg feed administered to the mother, corresponding to an exposure of 5.85 mg/kg b.w./day during gestation and 13.1 mg/kg b.w./d during lactation (given the difference in weight of mothers before and after parturition). The dose of 5.85 mg/kg b.w./d is very close to the dose of 5 mg/kg b.w./d on which the TDI is based.
The absence of effect on the developing nervous system led to the conclusion that the product is not neurotoxic in the range of doses tested, within the meaning of OECD Guideline 426.
Comments of the BPA WG:
- The study was conducted according to OECD Guideline 426 (tests, histopathological examinations, etc.) in accordance with good laboratory practice.
- The dose range selected was sufficiently broad. The step between each level of low (x10) and high doses (x3) was appropriate. However, the step between the intermediate doses (1.5 and 75 mg/kg) could be subject to criticism (x50) with respect to the recommendations of the OECD guideline.
- The lack of a positive control group in the study was not detrimental because all the tests and automated equipment used had been validated with reference substances as part of studies that were completed between May and September 2008 (the experimental part of the study on BPA was conducted between July and November 2008).
- The BPA WG regrets the lack of toxicokinetic groups which, although not required by OECD Guideline 426, would have clarified the internal exposure of mothers, foetuses and newborns.
- Contrary to the authors, who found no effect on the physical development of offspring, the BPA WG noted a reduction of 5.6% in the pre-weaning mean weight at PND14-17 at 750 mg/kg of feed and 8.3% at PND11-21 at 2250 mg/kg of feed.
- In the supplementary study, the dose-response relationship was not investigated (only one dose tested, 2250 mg/kg feed) and it would have been interesting at least to have tested again the dose at 750 mg/kg feed. In addition, measurements of the plasma concentration could have helped to interpret the results.
- The control of experimental conditions was insufficient both in the main study and in the supplementary study, mainly because of the high concentration of phytoestrogens in the diet (312-333 mg/kg of isoflavones).

***To conclude, the BPA WG** accepts the lack of neurotoxicity of BPA, provided that these findings be substantiated by further study with multiple doses around that of 5 mg/kg b.w./day, focused on investigating the occurrence of convulsions, with careful control of the experimental conditions (feed, bedding), and including measurement of plasma concentrations in treated mothers and offspring.*

3.1.2 *In utero* and lactational exposure to bisphenol A, in contrast to ethinyl estradiol, does not alter sexually dimorphic behavior, puberty, fertility and anatomy of female LE rats

Ryan B.C., Hotchkiss A.K., Crofton K.M., Gray E.A. *Toxicological Sciences* (2010) 114: 133-48.

Rating 2c

Study objective:
To establish whether perinatal exposure *(in utero* and during lactation) to low doses of ethinyl estradiol (EE_2) and BPA disrupts behaviour related to sexual dimorphism, age at puberty and reproductive function in Long Evans (LE) rats.
Method:
- Gavage of two groups of pregnant females each comprising groups of 13-29 females/dose (group 1) and 6-14 females/dose (group 2), from the 7th day of gestation (GD7) until weaning of pups (PND18), with corn oil (control), ethinyl estradiol (0.05 to 50 µg/kg with four dose levels for group 1 and seven for group 2) or BPA (2, 20 or 200 µg/kg with three dose levels for group 1 and two for group 2).
- Parameters measured on the F1 females:
  - at PND2: sex, body weight, anogenital distance.
  - from PND23: vaginal opening.
  - after weaning: fertility and fecundity (for 4 months), preference for saccharin (test on group 1 for 5 days), activity (before and after administration of EE_2 for 14 days), sexual receptivity (lordosis) after bilateral ovariectomy and administration of 50 µg/kg of EE_2 and subcutaneous injection of 0.5 mg of progesterone.
  - Morphometry of the external genitalia (length and depth of the urethral slit, urethral-vaginal distance, anogenital distance).

Results:
No effect of BPA on the parameters studied, whereas pre- and neonatal exposure to EE_2 caused the following effects:

- drop in body weight of mothers (at doses ≥ 15 µg/kg)
- drop in implantations, number and body weight of offspring at PND2 (50 µg/kg)
- precocious puberty (5 µg/kg)
- drop in the fertility of F1 females
- drop in the preference for saccharin of F1 adult females
- suppression of sexual receptivity at doses of 15 and 50 µg/kg not re-established by the administration of EE_2 and progesterone

Study conclusion:
Pre- and neonatal exposure to low doses of BPA (2-200 µg/kg b.w., or 40-4000 times higher than average exposure levels of the U.S. population estimated by NHANES, theNational Health and Nutrition Examination Survey) did not induce any significant effect on the weight of mothers and offspring, primary sexual characteristics (anogenital distance, vaginal opening, morphology of external genitalia), fertility, fecundity and behavioural sexual dimorphism, thus confirming the results of multi-generation studies (including those of Cagen *et al.*, 1999; Ema *et al.*, 2001; Tinwell *et al.*, 2002 and Tyl *et al.*, 2002).

Comments of the BPA WG:

- The study protocol was well described and approved by the U.S. EPA. The duration of treatment covered most of the period of sexual differentiation. The range of doses for the positive control and BPA was appropriate and the differences between doses were acceptable (up to x10, as recommended by OECD). Tests and methods have been properly validated. It was particularly interesting to note that the reference product, ethinyl estradiol, a synthetic estrogen, responded positively in this study.

- This study was conducted in Long Evans rats, which some authors consider to be insensitive to estrogen (Vom Saal, 2008). To justify the use of the Long Evans rat model, the authors made some comparisons with the Sprague Dawley rat including demonstrating a similar response to the sexual receptivity test (ovariectomy/EE_2 and progesterone treatment) and a comparable dose-response relationship between the LE and SD strains in terms of uterine weight of ovariectomised females after treatment with EE_2 (0.5 – 250 µg/kg).

- It was not possible to verify, from the tables of results, the exact number of animals studied in the different groups, nor therefore to assess the power of the study to detect different effects (in this respect, the authors themselves noted that the number of animals used to detect the effects on fertility/fecundity was restricted). The results were nevertheless sufficiently detailed to allow an interpretation to be made. Note that some of these results (maternal toxicity and fertility of the F0, effect on F1 males) were merely a reiteration of previously published work.

- The BPA WG regrets the lack of plasma assays that would have specified the perinatal exposure of rats.

- There was regular monitoring of pesticides and heavy metals in the drinking water but no mention of the monitoring of BPA levels.

- The animals were housed in polycarbonate cages.

- The animals' diet contained high levels of phytoestrogens (around 400 mg/kg of isoflavones according to data from the supplier).

- The periodicity of the light conditions was unconventional (14h of light and 10h of night) which might influence the behavioural analyses.

- Note: the occurrence of effects in males was not investigated in this study. They were published by Howdeshell et al. (2008) only for parameters related to reproduction.

Despite the few reservations outlined above, the BPA WG accepts the results of this study that showed no effects of BPA.

***To conclude, the BPA WG** accepts the lack of toxicity of BPA in this study of primary sexual characteristics (anogenital distance, vaginal opening, morphology of external genitalia), fertility, fecundity and behavioural sexual dimorphism in female rats.*

3.1.3 Gestational and lactational exposure to ethinyl estradiol, but not bisphenol A, decreases androgen-dependent reproductive organ weights and epididymal sperm abundance in the male Long Evans Hooded rat

Howdeshell K.L., Furr J., Lambright C.R., Wilson V.S., Ryan B.C., Gray Jr L.E. *Toxicological Sciences* (2008) 102: 371–382.

Rating 2c

Study objective:
To establish whether perinatal exposure *(in utero* and during lactation) to low doses of ethinyl estradiol (EE_2) and BPA disrupts reproductive function in male Long Evans (LE) rats.
Method:
Gavage of Long Evans rats with 0.2, 20 and 200 µg/kg/day of BPA or 0.05 and 50 µg/kg/day of EE_2 (ethinyl estradiol) from GD7 to PND18 (from the 7th day of gestation to the 18th day after birth).
Parameters measured: anogenital distance at PND2 (2nd day after birth) and persistence of nipples at PND14 (14th day after birth).
Euthanised at PND150 (150th day after birth) or at PND229 and hormonal measurements taken (estradiol, testosterone, corticosterone, T4, LH and PRL) with semen analysis and histopathology of sex organs.
Results:
No significant effect of BPA was observed on the parameters studied, in contrast to control animals treated with ethinyl estradiol.
Comments of the BPA WG:
- The BPA WG accepts the findings of this study that do not show any effects of BPA at the low doses tested. The effects observed with the positive control (EE_2) show the reliability of the model and the protocol used and the sensitivity of the Long Evans strain of rat (results were similar to those obtained in studies performed in Sprague Dawley and Wistar rats).
- The BPA WG regrets the lack of plasma assays that would have specified the perinatal exposure of rats.
- There was regular monitoring of pesticides and heavy metals in the drinking water but no mention of the monitoring of BPA levels.
- The animals were housed in polycarbonate cages.
- The animals' diet contained high levels of phytoestrogens (around 400 mg/kg of isoflavones according to data from the supplier).
- The periodicity of the light conditions was unconventional (14h of light and 10h of night) which might influence the behavioural analyses.
- Note: the occurrence of effects in females was not investigated in this study. They were published by Ryan et al. (2010).
- The BPA WG regrets the lack of measurement of plasma concentrations.

***To conclude, the BPA WG** accepts the lack of toxicity of BPA on the primary sexual characteristics (anogenital distance, morphology of external genitalia) and reproductive functions in male rats.*

3.2 Studies considered as warning signals

3.2.1 Alterations in male infant behaviors towards its mother by prenatal exposure to bisphenol A in cynomolgus monkeys (*Macaca fascicularis*) during early suckling period

Nakagami A., Negishi T., Kawasaki K., Imai N., Nishida Y., Ihara T., Kuroda Y., Yoshikawa Y., Koyama T., *Psychoneuroendocrinology* (2009) 34: 1189-1197.

Rating 2e

Study objective:
To assess the effect of perinatal exposure *(in utero* and during lactation) to BPA on neurobehavioural development in nonhuman primates so as to consolidate the extrapolation to humans of observations on rodents that show altered behavioural sexual dimorphism after perinatal exposure to BPA.
Method:
- Subcutaneous administration of 10 µg of BPA/kg b.w./day delivered by osmotic pump (changed every 28 days) to 18 females (plus 19 controls receiving the vehicle) from the 20th day of gestation (GD20) to the 160th postnatal day (PND160), to obtain a blood level equivalent to an intake of 5 mg/kg/day in rats.
- Comparison of mother-child behavioural interactions (4-5 males, 6-10 females) at PND 31-60 and 61-90 by biweekly videotaping of maternal (14) and infant (14) behavioural variables for ten minutes.
- Data exploitation by univariate and multivariate analysis (to determine the discriminant scores).
Results:
- Univariate analysis: significant effect of BPA on three infant behaviours and one maternal behaviour:
  - Among male infants: "clinging" and "social exploration" behaviours were reduced at the age of 2 months and “outward looking” behaviour was increased at 2 and 3 months.
  - Among male-nursing mothers , “outward looking” behaviour was increased at the ages of 2 and 3 months.
- Multivariate Analysis (PCA): after administration of BPA, the discriminant scores of male infants were more like those of the female infant controls than those of the male infants in the control group. In contrast, no effect was observed in females. Regarding maternal behaviour, male-nursing mothers exposed to BPA had discriminant scores closer to those of control female-nursing mothers than those of control male-nursing mothers.

Study conclusion:
The authors concluded that the "outward looking" behaviour was the one most affected by BPA in male infants and male-nursing mothers. BPA appeared to have an effect on the sexual differentiation of the mother/child behavioural interaction, in the sense of a feminisation of male infants.
The cynomolgus monkey model would more effectively highlight the effects of endocrine disruptors, particularly BPA (compared to the rat or mouse rodent model).
Comments of the BPA WG:
- Exposure to BPA: the plasma levels of BPA measured in mothers only at the 50th day of gestation were below the detection limit (12.5 ng/mL); no measurement was available for the infants. The difference in metabolism according to the route of administration was not taken into account. No dose-response relationship can be established (a single dose was tested).
- Interpretation: only 1-3 variables were modified out of 14 in the short-duration recordings (10 minutes) of monkey behaviour. Their significance remains to be established, particularly since, as the authors pointed out, no explanation in terms of psychological impact can be given for the results.
The route of administration (non-oral), the lack of a positive control, the lack of data on the actual exposure of the infants, on the phytoestrogen levels in the feed and BPA levels in the water, the fact that just one dose was tested and the significance of the observed effects make interpretation of this study difficult.

***To conclude, the BPA WG** nevertheless interprets the results of this study as a warning signal.*

3.2.2 Effects of developmental exposure to bisphenol A on brain and behavior in mice

Palanza P., Gioiosa L., vom Saal F.S., Parmigiani S., *Environmental Research* (2008) 108: 150–157.

Rating 2g

This article is a meta-analysis of this team’s work on CD-1 mice, on the neurological and behavioural effects of BPA, with a single oral dose of 10 µg/kg/day.

- **Maternal behaviour**

The behaviour of the F1 generation (15 females per group) was examined after administration of BPA under 3 scenarios:

1) during gestation only (from the 14th to the 18th day of gestation)
2) during gestation and continued after birth until adulthood
3) only after birth until adulthood.

Results:

Changes in the maternal behaviour of mice from the F1 generation only appeared when they were exposed either *in utero* or in adulthood (scenarios 1 and 3), but not after the two periods of combined exposure (scenario 2). These changes corresponded to a reduction in the time that mothers spent with their offspring and an increase in the time they were alone in the cage (isolated rest time). Nevertheless, this had no effect on the weight of offspring at birth, suggesting that the level of care provided by these mothers was adequate.

Comments of the BPA WG:

The significance for human health of the observed effects was not established (time that the mice spent with their offspring). The authors did not explain why the BPA - BPA sequence led to no change (apart from the increase in the isolated rest time for the mother).

- **Exploratory and emotional behaviour**

The behaviour of the offspring of treated mothers was assessed for the following situations and ages:

- reaction test to a new environment before puberty (between 28th and 30th postnatal day, PND 28-30), mothers treated from GD11 to PND7 (from the 11th day of gestation to the 7th day after birth)
- open field exploration test in adulthood; mothers treated from GD11 to GD18 (from the 11th to 18th day of gestation)
- maze test in adulthood, mothers treated from GD11 to PND7.

Methoxychlor (a pesticide with recognised estrogenic effects) was used as a positive control (dose of 20 µg/kg/day).

Results:

Exposure to a dose of 10 µg/kg/day from GD11 to PND7 altered exploratory behaviours and anxiety in adulthood (open field, maze) and curiosity (before puberty), which were sexually differentiated in controls (Gioiosa *et al.*, 2007[3]).

Comments of the BPA WG:

This study suggests persistent effects after perinatal exposure to BPA on sexual behaviour of the brain. However, the significance for human health of the observed effects was not established (curiosity, anxiety). It is unfortunate that just one dose was used. If these results are to be taken into account in the context of a health risk assessment, they should be confirmed by a new study conducted with several doses.

- **Brain dopamine function**

Method:

This part of the publication summarised the studies of the effect of prenatal exposure to BPA on the development of brain dopamine function. Mice (3 per sex) were administered BPA orally from GD11 to GD18 and were tested for CPP (Conditioned Place Preference) induced by injection of amphetamine at PND60 (adult).

Results:

Treated females (foetal exposure) did not exhibit the amphetamine-induced conditioning observed in control females.

Comments of the BPA WG:

These results were discussed in relation to previous results, suggesting an estrogenic effect on brain development (e.g. gender differences in the volume and number of cells in the locus coeruleus in the brain) and particularly on the establishment of the monoaminergic system.

- ***Comments on this meta-analysis by the BPA WG***

The relevance of this meta-analysis relates to the comprehensive study of a low dose of BPA according to the window of exposure, in experimental conditions that were very similar since they were performed by the same team.

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[3] Gioiosa L., Fissore E., Ghirardelli G., Parmigiani S., Palanza P. (2007). Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice. *Horm. Behav*. 52: 307-16

The behavioural effects appeared to be very sensitive, but just one single dose and one single species were studied. The lack of a positive control, the lack of data on the actual exposure of the offspring, on the phytoestrogen levels in the feed and the endocrine disruptors in the bedding (corn cobs), the fact that just one dose was tested and the significance of the observed effects make interpretation of these studies difficult.

***To conclude, the BPA WG** nevertheless interprets the results of this meta-analysis as a warning signal.*

3.2.3 Work by Salian *et al.* on the male reproductive system after perinatal exposure to BPA

a) Impairment in protein expression profile of testicular steroid receptor coregulators in male offspring perinatally exposed to bisphenol A
Salian S., Doshi T., Vanage G., *Life Sciences* (2009) 85: 11-18.

Rating 3

Study objective:
To determine whether impaired spermatogenesis related to perinatal exposure to BPA is associated with altered expression of steroid receptors and their cofactors in the testis.
Method:
- Administration by gavage of 1.2 or 2.4 µg/kg b.w./day of BPA in sesame oil to groups of eight female rats (Holtzman strain) from the 12th day of gestation (GD12) to the 21st day after birth (PND21).
- Litters reduced to 5 males reared until weaning, then selection of 24 males/group at PND75 for the constitution of an F2 generation (same for F3).
- Immunohistochemical analyses of receptor coregulators (SRC-1, GRIP-1, p/CIP and NCoR) in sections of testes (3 per individual, or 72/group/generation).
Results:
- Effects were observed at both doses in the three generations (F1, F2 and F3), whereas only the F0 females were exposed during gestation and lactation:
  - decrease in SRC-1 expression in the tubules at stage III
  - increase in GRIP-1 expression in the tubules at stage IX and in elongated spermatids
  - increase in p/CIP expression in the early stages (only at the low dose for F2 and F3)
  - reduction in NCoR expression in Leydig cells (nucleus and cytoplasm).
Study conclusion:
Disruption to the expression of steroid receptor coregulators induced by BPA could potentially affect the germ line for several generations and have important implications for fertility.
Comments of the BPA WG:
- Clearly, the role of steroid receptor cofactors has not yet been well established, as is demonstrated, for example, by some knockout mice models for GRIP-1 and NCoR. The authors therefore provided a cautious interpretation of the observed variations and formulated assumptions about the mechanisms of a trans-generational effect.
- The BPA WG noted the choice of dose based on unpublished results, the failure to justify the choice of strain, the lack of data on the declared decline in fertility for the three generations (correlation not possible with the immunochemical results), the lack of hormonal assay, the lack of spermogram and semen analysis. This study suggested a trans-generational effect which must be verified.
*The lack of a positive control, the lack of data on the actual exposure of offspring, on the level of endocrine disruptors in the bedding (*paddy husks*), the fact that the rats' feed was prepared by the laboratory (non-standardised diet), the absence of a dose-response relationship and the significance of the observed effects make interpretation of this study difficult.*

***To conclude, the BPA WG** nevertheless interprets the results of this study as a warning signal.*

b) Perinatal exposure of rats to Bisphenol A affects the fertility of male offspring
Salian S., Doshi T., Vanage G., Life Sciences (2009) 85: 742-752.

Rating 3

Study objective:
To establish the effects of perinatal exposure to BPA in male rats on fertility parameters and the expression of SR receptors in adulthood (study of 3 generations).

Method:
Female Holtzman F0 rats received by gavage either BPA (1.2 and 2.4 µg/kg b.w./day) or diethylstilbestrol (DES, 10 µg/kg b.w./d) from the 12th day of gestation (GD12) to the 21st day after birth (PND21), 8 F0 mothers/dose.
Litters were reduced to 4-5 males at birth, then the offspring were reared, before selection of 24 males/group at PND75.
Fertility was assessed on F1, F2 and F3 (n=24 males/group). Only the F1 generation was exposed until weaning. The steroid receptors (SR) were localised in the testes (by immunohistochemistry) of the F1, F2 and F3 generations at PND125.
Body weights, weights of reproductive organs, sperm count and motility, and hormone concentrations (F1) were also measured at PND125.
Results:
- DES, the positive control, had a trans-generational effect on fertility: it increased the time required for mating and strongly decreased the number of mated females (only 25-30% of the females mated). BPA did not affect the mating index (the figure of over 80% of mated females was not significantly different from the controls), but it increased the time required for mating. This was observed over several generations.
- For DES, pre- and post-implantation losses were increased greatly in all the generations. For BPA, pre-implantation losses were significantly increased only at 2.4 µg/kg b.w. in the F3 generation and post-implantation losses were significantly increased only in the F3 generation at both doses.
- The litter size of females mated with males from F1, F2 or F3 was reduced after exposure to BPA or DES, more significantly for DES.
In the offspring (at the 125th day after birth), a trans-generational increase in body weight was reported in the groups exposed to BPA (except F1 at 2.4 µg/kg b.w.). No effect on the relative weight of reproductive organs was identified with BPA.
The decrease in sperm count and motility was comparable to that with DES, whereas testicular degenerative lesions were described in the DES group only (a few specific lesions were described with BPA, unrelated to the treatment).
Hormone assays (LH, FSH, E_2 and testosterone) conducted only on F1 males showed a significant decrease in concentrations of all hormones tested (except LH at the high dose). This decrease was not proportional to the dose of BPA.
The immunochemical analysis showed decreased expression of Sertoli cell androgen receptors in all the generations at the low dose (1.2 µg/kg b.w./d), but only in the F3 generation at the high dose (2.4 µg/kg b.w./d) as well as decreased expression of estrogen receptors ERβ (at both doses) in all generations and an increase in ERα in F1 (at both doses).
Study conclusion:
This study showed a trans-generational effect on male fertility and testicular steroid receptor expression after perinatal exposure to BPA at low doses. This effect would be mediated by the impaired germ line.
Comments of the BPA WG:
The results did not enable a dose-response relationship to be established. It would have been preferable to compare the hormonal changes with those induced by DES in F1 and to have the results for the F2 and F3 generations (spermogram/hormonal changes correlation). In addition, the immunohistochemical results should be interpreted with caution, especially for ERα (sensitivity and reproducibility of the measurement); results with DES would be required.
The lack of data on the actual exposure of the offspring, on the levels of endocrine disruptors in the bedding (paddy husks), the fact that the rats' feed was prepared by the laboratory (non-standardised diet) and the absence of a dose-response relationship make interpretation of this study difficult.

***To conclude, the BPA WG** nevertheless interprets the results of this study as a warning signal.*

c) Neonatal exposure of male rats to bisphenol A impairs fertility and expression of sertoli cell junctional proteins in the testis

Salian S., Doshi T., Vanage G., *Toxicology* (2009) 265: 56-67.

Rating 3

Study objective:
To study the effect of exposure to BPA in the first few days of life on fertility and expression of Sertoli cell junctional proteins (SCJP) in male rats.

Method:
Male rats (Holtzman, n=24) were exposed to BPA by subcutaneous injection at doses of 0.6 to 10 µg/rat (100, 200, 400, 800 and 1600 µg/kg b.w.) for the first 5 days after birth (PND1 to 5). Diethylstilbestrol (DES) was used as a positive control (1600 µg/kg b.w.).
Male fertility was assessed in adulthood. At PND75, males mated with females; one group of females was sacrificed on the 20th day of gestation and another at parturition. At PND125, an immunohistochemical analysis of Sertoli cell junctional proteins (SCJP) was carried out on sections of testes of rats exposed to the doses of 400 µg/kg b.w. (determined as the lowest dose to impair fertility) and 1600 µg/kg b.w. In parallel, for the dose of 400 µg/kg b.w., SCJP were localised at PND 15, 30, 45 and 90.
Results:
A significant increase in post-implantation loss, reduced litter size, changes in sperm count and motility, as well as changes in hormone levels were observed after treatment of males in the neonatal period. These effects occurred from the dose of 400 µg/kg b.w., which was identified as the lowest observed adverse effect level (LOAEL). Changes in Sertoli cell junctional protein expression were observed (decrease in Cx-43, increase in N-cadherin and Zo-1) at this dose and at 1600 µg/kg b.w.
Study conclusion:
Exposure to BPA in the first few days of life of the male rat altered fertility. As changes in Sertoli cell junctional protein expression may contribute to this effect, the authors proposed that these proteins be considered as biomarkers of the testicular effect of BPA and other estrogenomimetic compounds.
Comments of the BPA WG:
The route of administration was subcutaneous, making interpretation of this study difficult. The results did not enable a dose-response relationship to be demonstrated on all the measured parameters (resorption, litter size, hormone levels). The testicular lesions were not studied for the dose of 800 µg/kg b.w. Concerning the changes in junctional protein expression, it would have been interesting to analyse a group treated with DES or another reference estrogen. The authors controlled for potential bias by using polypropylene cages and a soy-free diet.
The route of administration, the lack of data on levels of endocrine disruptors in the bedding (paddy husks), the fact that the rats' feed was prepared by the laboratory (non-standardised diet) and the lack of a dose-response relationship make interpretation difficult.

***To conclude, the BPA WG** nevertheless interprets the results of this study as a warning signal.*

d) *Comments of the BPA WG on the 3 studies by Salian et al. (2009)*

The authors concluded that they have identified trans-generational effects on fertility and testicular steroid receptor expression after perinatal exposure of males. Some inconsistencies have been compiled below, which prevent the results of this study from being used directly to assess the risks to human health.

- *The authors demonstrated an alteration of the time required for mating in F1, F2, F3 for all of the treated groups while the copulation index was not affected for BPA (number of females mated).*
- *The authors reported an increase in post-implantation losses in F3 for all the treated groups, while litter size in the BPA groups was reduced for all generations.*
- *Regarding the body weight of the offspring, increases for F1, F2 and F3 were reported, except for BPA in F1, at 2.4 µg/kg b.w./d.*
- *Sperm count and motility seemed similarly reduced between BPA and DES for all generations, while testicular histological lesions concerned only the DES groups.*

The immunohistochemical analysis also yielded some inconsistent results: a trans-generational reduction in the expression of androgen receptors at 1.2 µg/kg b.w./d for the three generations and only in F3 at 2.4 µg/kg/d. With regard to the increased expression of ERα in F1 and the trans-generational reduction in ERβ, the sensitivity and reproducibility of the measurements should be ensured and results with DES should be provided.

***To conclude, the BPA WG** nevertheless interprets the results of these three studies by Salian et al. (2009) as a warning signal, without proven significance for human health.*

3.2.4 Impact of oral bisphenol A at reference doses on intestinal barrier function and sex differences after perinatal exposure in rats

Braniste V., Jouault A., Gaultier E., Polizzi A., Buisson-Brenac C., Leveque M., Martin P.G., Theodorou V., Fioramonti J., Houdeau E., *Proceedings of the National Academy of Sciences USA* (2010) 107: 448-453.

Rating 2e

Study objective:
To investigate the effects of BPA on intestinal permeability in ovariectomised females and young rats after perinatal exposure.
Method:
The experiments were conducted:

1) on groups of 5-15 ovariectomised female Wistar rats fed a standard diet and receiving by gavage BPA in corn oil for 15 days (0, 0.05 and 5 mg/kg, positive control = 0.6 mg/kg of estradiol benzoate in corn oil). During the final 5 days of administration, a daily subcutaneous injection of 2 mg/kg of an ER antagonist (ICI 182,780) was performed in 3 groups receiving BPA at 5 mg/kg and in the positive control group treated with estradiol benzoate.
2) on groups of 8-13 female rats receiving by daily gavage 5 mg/kg of BPA in corn oil during gestation and lactation (GD15 to PND21). At weaning, the offspring were fed the standard diet.

Intestinal permeability (Ussing chambers) and intestinal inflammation (myeloperoxidase) caused by intracolonic administration of TNBS (trinitrobenzene sulfonic acid) were measured. Sensitivity to visceral pain was also measured by electromyography in ovariectomised rats subjected to colorectal distension, which induces abdominal contractions whose intensity enables the visceral motor response to be assessed.
Results:
BPA decreased intestinal permeability, in a dose-dependent manner (two doses tested), in ovariectomised females. This effect, similar to that produced by estrogen, is dependent on a sealing of the tight junctions in the intestinal epithelium. BPA at the dose of 5 mg/kg also reduced the severity of intestinal inflammation and increased sensitivity to visceral pain. In rats whose mothers were treated with 5 mg/kg of BPA, decreased intestinal permeability and increased inflammatory response were observed, only in females.
Comments of the BPA WG:
The results showed effects in the intestine of the offspring, but they were essentially only based on a single dose (5 mg/kg b.w./d). The experimental protocol aimed to explore the mechanisms of action and not to highlight a dose-response relationship. The article discussed the effects associated with changes in intestinal permeability during the first few days of life. The effect on adults was observed in ovariectomised animals.
The lack of data on the actual exposure of offspring, on the level of phytoestrogens in the diet and endocrine disruptors in the bedding, on BPA in the water, the lack of information on the nature of the cages, the fact that just one dose was tested during perinatal exposure and the significance of the observed effects make interpretation of this study difficult.

To conclude, the BPA WG *nevertheless interprets the results of this study as a warning signal.*

3.2.5 Prenatal exposure to bisphenol A at environmentally relevant doses adversely affects the murine female reproductive tract later in life

Newbold R.R., Jefferson W.N., Padilla-Banks E., *Environmental Health Perspectives* (2009) 117: 879-885.

Rating 2e

Objectives:
To study whether prenatal exposure of mice to BPA can cause long-term effects in the tissues of the female reproductive system.
Methods:
Female CD-1 mice were treated from the 9th to the 16th day of gestation (GD9-16) at doses of 0.1, 1, 10, 100 and 1000 µg BPA/kg b.w./d (5 mothers/dose) by subcutaneous injection. A histological analysis was conducted on the genitalia of F1 females, removed once they had reached adulthood (16-18 months).

Results:
A high frequency of ovarian cysts was observed in all treated groups but this was statistically significant only for the dose of 1 µg BPA/kg b.w./d.
Similarly to what has been reported for diethylstilbestrol (DES) in other studies, increased progressive proliferative lesions of the oviduct after BPA treatment was highlighted.
In some treated animals, atypical hyperplasia and stromal polyps of the uterus, sarcoma of the uterine cervix, and mammary adenocarcinomas were found. The females in the group treated with 0.1 µg BPA/kg b.w./d had the highest rate of tumours in the reproductive tract (36% of treated mice).
Study conclusion:
The authors suggested that BPA administered at critical periods of differentiation may in the long-term cause benign or malignant tumours of the female reproductive system.
Comments of the BPA WG:
This study was based on exposure to BPA during a critical window of gestation and identified greater effects at low doses (0.1 and 1 µg/kg b.w./d). Some experimental conditions were controlled (diet, cage) but not the bedding (hardwood chips) or the water bottles, which were polycarbonate. Moreover, the subcutaneous administration of BPA and the lack of a positive control make interpretation of this study difficult.

***To conclude, the BPA WG** nevertheless interprets the results of this study as a warning signal.*

3.2.6 Induction of mammary gland ductal hyperplasias and carcinoma in situ following fetal bisphenol A exposure

Murray T.J., Maffini M.V., Ucci A.A., Sonnenschein C., Soto A.M., *Reproductive Toxicology* (2007) 23: 383-390.

Rating 2e

Study objective:
To determine whether *in utero* exposure to very low doses of BPA may lead to deleterious effects on the development of the mammary gland or promote the occurrence of breast diseases during adult life.
Method:
Female Wistar rats received continuous subcutaneous administration (osmotic pump) of 2.5, 25, 250 and 1000 µg/kg b.w./d of BPA from the 9th day of gestation until parturition. Sacrifices were made at the 50th day (PND50, puberty) and at the 95th day after birth (PND95, adulthood) for a histological analysis of the mammary glands. The estrogenicity of diet and drinking water were verified as well as the cages and bedding (E-Screen assay).
Results:
- The reproductive parameters measured (litter size, weight of animals from the 1st to the 110th day after birth, sex ratio, age of vaginal opening, anogenital distance) indicated no difference between treated and control rats with the exception of the anogenital distance (PND4) in males exposed to the dose of 250 µg/kg b.w./d.
- intraductal hyperplasia in the mammary glands was observed from the lowest dose at PND50 and PND95. This hyperplasia also led to increased expression of the ERα receptor and the Ki67 antigen, which is a proliferation marker. *In situ* carcinomas were observed in 33% of the animals at PND95 for the two highest doses.
Comments of the BPA WG:
These results confirm and clarify a previous study by the same team conducted in mice[4].
Several doses were tested, the protocol was rigorous, the estrogenic potential of the diet, cages and bedding were assessed, the drinking water was contained in glass bottles. However, the study did not reveal a clear dose-response relationship. Moreover, subcutaneous administration of BPA and the lack of a positive control make interpretation of this study difficult.

***To conclude, the BPA WG** nevertheless interprets the results of this study as a warning signal.*

[4] Munoz-de-Toro M., Markey C.M., Wadia P.R., Luque E.H., Rubin B.S., Sonnenschein C., Soto A.M. (2005). Perinatal exposure to bisphenol-A alters peripubertal mammary gland development in mice. *Endocrinology* 146: 4138-4147.

3.3 Studies whose results were not considered by the BPA WG to be of concern and whose findings have not been accepted as a warning signal

3.3.1 Effects of neonatal exposure to bisphenol A on steroid regulation of vascular endothelial growth factor expression and endothelial cell proliferation in the adult rat uterus

Bosquiazzo V.L., Varayoud J., Muñoz-de-Toro M., Luque E.H., Ramos J.G., *Biology of Reproduction* (2009) 82: 86-95.

Rating 3a

Study objective:
To determine whether exposure to BPA during the first few days of life affects the uterine response to steroid stimuli.
Method:
- Subcutaneous injection of 0.05 or 20 mg/kg b.w./day of BPA or 0.2 µg/kg b.w./d of DES (diethylstilbestrol, positive control) in female rats after birth (at PND1, 3, 5 and 7). At the age of 80 days (PND80), the animals were ovariectomised (OVX) and then subjected to hormone replacement therapy (progesterone (P) + estradiol (E2)).
- Measured parameters: expression of VEGF (Vascular endothelial growth factor) extracted from uterine horns (PCR), immunohistochemistry for the expression of estrogen receptors ERα and co-repressor NCOR1, and quantification of endothelial proliferation by image analysis.

Results:
- Increased expression of VEGF in uterine endothelial cells in female OVX + E2 rats (verification of the model).
- Correlation between VEGF expression and endothelial proliferation.
- Decreased expression of estrogen receptors ERα at the low dose of BPA (0.05 mg/kg/day).
- Increased expression of co-repressor NCOR1 at both doses of BPA, associated with the change in VEGF at the lowest dose (0.05 mg/kg/day).

Study conclusions:
In young female rats exposed shortly after birth to low doses of BPA or DES and then ovariectomised in adulthood, uterine endothelial proliferation and VEGF expression were decreased following stimulation by ovarian steroids (P+E2). As VEGF is essential for implantation, the authors suggested an adverse effect on fertility of low doses of BPA or DES.
Comments of the BPA WG:
The data are insufficient for conducting a critical analysis, particularly with regard to the lack of details on the experimental protocol. Moreover, the subcutaneous administration of BPA does not allow these results to be extrapolated to the oral route.

***To conclude, the BPA WG** does not accept the conclusions of the authors, because of the methodological limitations.*

3.3.2 Prenatal bisphenol A exposure and early childhood behavior

Braun J.M., Yolton K., Dietrich K.N., Hornung R., Ye X., Calafat A.M., Lanphear B.P., *Environmental Health Perspectives* (2009) 117 (12): 1945-1952.

Rating 3b

Study objective:
To study the association between prenatal exposure to BPA (measured by urine concentration at 16 and 26 weeks of gestation and at birth) and child behaviour at 2 years of age (measured by the BASC-2 behavioural test).
Method:
The study involved 249 mother-child pairs from Cincinnati (Ohio, USA) from a cohort recruited in 2003 to study exposure to environmental contaminants (including lead).
Maternal urine was collected at around 16 and 26 weeks of gestation and at birth. The urine from week 16 was in fact collected between week 11.2 and week 21.4. Urinary concentrations of BPA were analysed by HPLC/MS (total: free + conjugated BPA).
Children's behaviour was assessed at 2 years of age using the preschool version of the BASC-2 test, which uses questionnaires self-completed by parents to assign composite scores for externalising (hyperactivity and aggression) and internalising behaviour (depression, anxiety and somatisation), and for calculating the BSI (Behaviour Symptom Index) reflecting the overall level of problem behaviours. In principle, the tendency of parents to describe their children in an overly negative way is detected by

the test. Scores of 40-60 are considered normal, those of 60-69 as "at risk" and those equal to or above 70 as "clinically significant".
The association between BPA concentrations and behaviour was analysed by linear regression and the results analysed by quartiles of urinary concentrations of BPA for each gestation time (i.e. a breakdown of the urine results into 4 groups).
Results:
- Median BPA concentrations in urine were respectively 1.8 ng/mL, 1.7 ng/mL and 1.3 ng/mL at the 16th and 26th weeks of gestation and at birth. The mean BPA concentration for all measurements was 2 ng/mL (mean BPA concentrations for each gestation time were not indicated).
- Mean composite scores (externalising, internalising and BSI) were respectively 47.6 (standard deviation 7.8, range of scores 30-84), 44.8 (SD 7, range of scores 31-68) and 50.4 (SD 6.8, range of scores 34-74).
- The authors noted an association between urinary BPA concentrations (creatinine-adjusted) divided into quartiles at 16 weeks of gestation and the externalising/BSI score for all children, although the results indicated that the variation was significant only for girls (data per quartile was not shown). In fact, there was a positive association between the BPA concentration at the 16th week of gestation and externalising scores only when the measurement was actually taken before the 16th week of gestation (minimum at 11.2 weeks), without however the level of significance being indicated. No other variation between urinary BPA concentrations and test score was significant, whether for all children or by sex, and irrespective of the duration of gestation at the time of urine collection.
- Mean creatinine-adjusted BPA concentrations did not differ significantly before and after the 16th week.
- Further analyses of maternal serum concentrations of lead and cotinine and maternal IQ were not presented. According to the authors, these variables did not alter the conclusions of the study. The same is true for the inclusion of mothers for whom not all the urine samples were available.
Authors' conclusions:
Prenatal exposure to BPA may be associated with externalising behaviours in children of 2 years of age, particularly girls.
Comments of the BPA WG:
- Concerning the concentration of BPA in the urine of mothers:
- *The authors indicated that BPA concentrations in urine were stable for at least six months, even up to 30 months, yet some samples were stored for up to 4-5 years before analysis.*
- *Exposure was estimated by quantification of total BPA (sum of BPA in free form and its conjugated metabolites) measured on a single sample at 3 periods of gestation, which is irrelevant since the toxicokinetic aspects of BPA were not taken into account (half-life of BPA is only a few hours; the conjugated forms of BPA are not toxic). Data adjusted for the creatinine concentration indicated no difference between BPA concentrations at 16 or 26 weeks of gestation or at birth (described as having "little correlation", Pearson coefficient < 0.11).*

- Concerning the test results:
- *The BASC-2 test is commonly used to assess aspects of the behaviour of individuals in American populations in the preschool and school environment, and to monitor any changes in individual scores over time. It is normalised to that effect. It has versions for three age groups (2-5 years, 6-11 years and 11-22 years) with the last two being considered most relevant (Harlacher and Merrell, 2008). The BPA WG noted that the authors used the test at the extreme lower limit of the age of validity (2 years) referred to by PsyCorp, the company which markets it.*
- *The BASC-2 scores are normalised for the American population with a mean of 50 and a standard deviation of 10. The results described are therefore relative to an American perception of what constitutes normal behaviour.*
- *The highest mean score was 53.9 (SD 1.3). The study of associations between BPA concentrations and effects on behaviour therefore focused on variations that were within the norm of inter-individual variation.*
- *There was no information on individual values of BPA concentrations for mothers whose children obtained composite scores outside the norm.*
- *The findings regarding composite score, according to the quartile of average maternal BPA concentrations for each sample, were not supported by significant statistical values, apart from the association between maternal BPA concentrations at 16 weeks and the externalising score, as well as the BSI of girls. The mean values of scores by quartile for the association in question are nevertheless within the test's range of normality. Longnecker (2009) also*

considered that the data presented were insufficient to support a difference in behaviour due to BPA between girls and boys.

- *BPA concentrations measured "at the 16th week" of gestation are actually spread out (they were obtained during prenatal visits) between weeks 11.2 and 21.4, or across a range of more than 10 weeks. The authors used this amplitude to present an association with measurements that actually predate the 16th week. However, the already limited number of samples analysed overall (249) was thus reduced by half, weakening the study, and distributed data were not presented.*
- *There are many limitations to the study and some were advanced by the authors themselves, including the uncertainties related to the proper quantification of BPA and the postnatal exposure of children to BPA. No data on the diet of mothers in the study were shown, nor on potential exposure of children to other substances in the months following birth. This should be reconciled with the initial quality of the cohort, recruited for the study of environmental toxicants including lead in the home (a prerequisite for study participation was to live in accommodation built prior to 1978). Blood lead concentrations in mothers were taken into account (results not described), but not those of children. The authors noted that the sample was unrepresentative: families living in older housing and having higher levels of education and income than the general population. As the BASC-2 was normalised for the general American population, the scores were adjusted to account for some of these confounding factors, such as education or income, but not the quality of housing.*

***To conclude, the BPA WG** does not accept the conclusions of the authors, because of the methodological limitations.*

3.3.3 Neonatal exposure to bisphenol A alters reproductive parameters and gonadotropin releasing hormone signaling in female rats

Fernández M., Bianchi M., Lux-Lantos V., Libertun C., *Environmental Health Perspectives* (2009) 117: 757-762.

Rating 2e

Study objective:
To explore the effects of a low and a high doses of BPA during the first few days of life on reproductive parameters in female rats.
Method:
- Daily subcutaneous injections of castor oil (control) or BPA at a low dose (50 µg corresponding to 2.5-6.2 mg/kg b.w.) and a high dose (500 µg corresponding to 25-62 mg/kg) during the first 10 days of life of Sprague-Dawley rats.
- Parameters measured**:**
  - Three days after the end of treatment (13th day after birth):
    - LH and FSH plasma levels; baseline and after stimulation with gonadotropin-releasing hormone (GnRH) (*in vivo* measurements);
    - GnRH pulsatility on cells isolated from the hypothalamus (*ex vivo* measurements) from biopsies of rats treated with BPA;
    - LH and FSH production by pituitary cells cultured in the presence of GnRH *(in vitro* measurements);
  - from the 60th to the 120th day after birth: body weight and vaginal opening.
  - at the 120th day:
    - measurement of LH and FSH plasma levels; baseline and after stimulation with GnRH (*in vivo* measurements);
    - LH and FSH production by pituitary cells cultured in the presence of GnRH *(in vitro* measurements);
    - inositol phosphate production by cultures of pituitary cells from biopsies of rats treated with BPA after the addition of GnRH, GnRH antagonist (Cetrorelix) or a mixture of both to the culture medium *(in vitro* measurements);
    - activation of $ERK_{1/2}$ (extracellular signal-regulated $kinase_{1/2}$) in cultures of pituitary cells after stimulation with GnRH (10^{-7} M) (*in vitro* measurements).

Results:
- Three days after the end of treatment, the baseline LH plasma level and that after stimulation with GnRH was reduced only at the high dose of BPA. FSH was unchanged. GnRH pulsatility was increased at both doses but the interval between the peaks was greater.

- BPA caused early vaginal opening (dose-dependent effect on the basis of two doses tested). With the highest dose, adult females remained in permanent oestrus, reflecting a very distorted cycle.
- At PND120, baseline LH and FSH plasma levels *(in vivo)* were not affected by BPA. After stimulation with GnRH, only the high dose reduced the increase in LH. As at PND13, GnRH pulsatility was increased at both doses, but the interval between the peaks was greater. *In vitro,* GnRH had the same effects as *in vivo.* GnRH stimulated the production of inositol phosphate less after treatment with the high dose of BPA. Activation of $ERK_{1/2}$ in pituitary cells taken from treated animals and tested *in vitro* was reduced by both doses of BPA.

Study conclusions**:**
Treatment with BPA during the first few days of life alters the reproductive parameters in offspring and adults. It causes early maturation of the hypothalamic-pituitary complex and accelerated puberty, alters GnRH pulsatility in offspring and adults, and the pituitary response to GnRH in adults.

Comments of the BPA WG:
Interestingly, this study shows a comprehensive approach based on both in vivo and in vitro results and monitored animals over a long-term period. However, the number of animals used was small. As suggested by the authors, the assumptions should be verified.
Another weakness that limits the interpretation of this study in terms of health risk is the route of administration of BPA (subcutaneous injection). The authors controlled for potential biases related to the cages (metal) and bottles (glass), but provided no information on the presence of BPA in drinking water and food, nor on the presence of phytoestrogens in food.
Investigations of pituitary cells taken from treated animals and cultured for 5 days showed effects at low doses. Nevertheless, the limitations of the model (especially the culture time) limit the scope of these results.

***To conclude, the BPA WG** notes that most effects were only observed at the high dose, above the NOAEL of 5 mg/kg b.w./d.*

3.3.4 Formation of adducts by bisphenol A, an endocrine disruptor, in DNA *in vitro* and in liver and mammary tissue of mice

Izzotti A., Kanitz S., D'Agostini F., Camoirano A., De Flora S., *Mutation Research* (2009) 679: 28–32.

Rating 3b

Study objective:
To assess the sensitivity threshold of DNA-adduct detection by ^{32}P-postlabelling in an acellular system and evaluate the formation of DNA adducts in liver and mammary cells of female CD-1 mice that received BPA in drinking water (200 mg/kg body weight) for eight consecutive days.

Method:
- Administration of BPA in drinking water (tap water) corresponding to an exposure of 200 mg BPA/kg b.w./d to 5 adult mice for 8 days (or 5 control mice not exposed to BPA) then removal of the liver and mammary glands.
- Extraction of DNA either directly in the reagent *(in vitro)* or from liver or mammary cells (pooled samples for each of the two groups of 5 mice).
- The DNA adducts, detected by measuring ^{32}P, were quantified by calculating the ratio of the activity detected in DNA adducts and in normal nucleotides (without adducts). The results were expressed as number of adducts per 10^8 nucleotides (detection limit of 0.1 adduct for 10^8 nucleotides).

Results:
Administration of BPA to mice caused the formation of DNA adducts in the liver (levels 3-4 times higher than in the controls) and in mammary cells (4.7 times higher than in the controls). According to the authors, the reaction of BPA with calf thymus DNA in the presence of liver microsomal fractions led to the dose-dependent formation of DNA adducts. The authors concluded that their work supported the hypothesis of a potential role of BPA in breast carcinogenesis.

Comments of the BPA WG:
Several methodological weaknesses appear in this article, especially the detection method selected to characterise the DNA lesions in vivo. The baseline level of DNA adducts in the tissues of control animals, obtained from a single pool of cells, seemed high.

***To conclude, the BPA WG** believes that, without confirmation, these results are not of concern, as the level of adducts was very low while the administered dose was high (200 mg/kg b.w./d).*

3.3.5 Occupational exposure to bisphenol A (BPA) and the risk of Self-Reported Male Sexual Dysfunction

Li D., Zhou Z., Qing D., He Y., Wu T., Miao M., Wang J., Weng X., Ferber J.R., Herrinton L.J., Zhu Q., Gao E., Checkoway H., Yuan W., *Human Reproduction* (2010) 25(2):519-27.

Rating 3b

Study objective:
To investigate the effects of BPA on sexual activity in humans.
Method:
- Epidemiological study conducted in China with a cohort (2004-2008) of:
  - 230 occupationally exposed men (from one BPA production plant and three resin production plants),
  - 404 male controls from "several" plants (construction materials, water suppliers, textile, electronics, retail, etc.) from the same geographical area as the BPA production plant, where workers "are unexposed" (284 volunteers and 120 husbands of women working in these unexposed plants).
- The protocol included measurements of BPA levels in the workplace atmosphere, history of exposure, individual monitoring, an inventory of protective equipment, hygiene measures and a record of exposure to other products.
- The volunteers were divided into subgroups according to the criteria above. BPA measurements were performed for each individual at the workstation and if this was not possible, the average value for the workshop was used. The exposure measurement was expressed as a cumulative value. Urinary concentrations of BPA (spot) were measured in volunteers.
- Individual questionnaires incorporating *the International Index of Erectile Function Inventory* were completed by the respondents themselves.
- The analysis was performed by odds ratio with a confidence interval of 95%.

Results:
After adjusting for confounding factors (age, social class, tobacco and alcohol consumption, chronic illness, duration of exposure, etc.), persons exposed to BPA had a higher risk of sexual dysfunction (3-7 times). A dose-response relationship was shown except for sexual desire. These effects occurred from one year of exposure.
Comments of the BPA WG:
The samples of sub-population studied were small. Considering the questionnaire and the measurements taken (air concentrations and urine analysis), it is difficult to ensure that volunteers had not guessed the purpose of the test (effect of exposure to one or more chemicals). The BPA WG notes that a self-administered questionnaire (without interviewer) represents an increased risk of response bias.
Even if the findings prove correct, they could not be applied to persons not occupationally exposed to BPA.

***To conclude, the BPA WG** believes that these results need to be confirmed in and outside occupational exposure, to enable conclusions to be drawn in terms of human health.*

3.3.6 Neonatal exposure to bisphenol A alters estrogen-dependent mechanisms governing sexual behavior in the adult female rat

Monje L., Varayoud J., Munoz-de-Toro M., Luque E.H., Ramos J.G., *Reproductive Toxicology* (2009) 28(4): 435-442.

Rating 3a

Study objective:
To establish whether neonatal exposure to BPA alters hypothalamic functions controlling the sexual behaviour of adult female Wistar rats.
Method:
- Subcutaneous injection of 0 (corn oil), 0.05 or 20 mg/kg of BPA, every 48 hours from the 1st to the 7th day of life (PND1-7). The low dose is comparable to the reference dose (RfD) set by the US EPA. The internal dose would be of the same order of magnitude as that of oral administration, due to the low

metabolic capacities of younger animals (work by Taylor *et al.,* 2008[5], showing the equivalence of doses after oral exposure vs. other routes in the neonatal period)
- At PND85 bilateral ovariectomy (10 females/group).
- At PND100:
  - Estrogen stimulation (subcutaneous implantation of capsules containing 17β estradiol) in order to measure (by immunohistochemistry) expression of estrogen receptors ERα and progesterone receptors PR and their cofactors (co-activator SRC-1 and co-repressor REA) in two brain areas involved in sexual behaviour (median preoptic area and ventromedial nucleus).
  - Behavioural analysis after subcutaneous injection of 10 µg of estradiol benzoate and 500 µg of progesterone, administered respectively 24h and 4h before videotaping sexual receptivity (lordosis[6] quotient and lordosis rating) and proceptive behaviour (ear wiggling, hopping and darting) over 10 minutes or 10 mounts (5 females per group).

Results:
- Decreased ERα receptor expression in both brain areas at both doses. Decreased PR receptor expression in the ventromedial nucleus after administration of the low dose but not after administration of the high dose.
- Increased SRC-1 expression at the low dose in the preoptic area, but decreased expression in the ventromedial nucleus, still at the low dose. The high dose had no effect on SRC-1 expression irrespective of the brain area.
- Increased REA expression in the ventromedial nucleus at both doses, but no change in the preoptic area.
- No change in responsiveness (lordosis quotient and rating), but proceptive behaviour (ear wiggling, hopping and darting) was altered at both doses.

Study conclusion:
The study showed a permanent alteration to brain control of the estrogen response associated with neonatal exposure to BPA, and suggested that the mechanism of action involved an alteration in the expression of cofactors of the estrogen receptors ERα.

Comments of the BPA WG:
- Exposure to BPA: the authors stressed that the choice of doses was compatible with the reference doses, however, the blood kinetics was not discussed (the protocol for subcutaneous injections at 48-hour intervals was not stated).
- Interpretation of results: in general, the diagrams did not highlight any dose-effect relationship (the BPA WG noted that the results were achieved with only two doses) for any of the parameters measured except for ERα and REA expression which were modified at both doses, but only in the ventromedial nucleus. These results therefore suggest a difference in sensitivity according to the brain area. The behavioural tests were difficult to interpret (2 measurements at 2-week intervals in 5 animals), especially since only two variables, whose meaning remains to be established (hops and darts), were changed at both doses.

***To conclude, the BPA WG** does not accept the results of this study as significant.*

3.3.7 Environmental endocrine disruptors promote adipogenesis in the 3T3-L1 cell line through glucocorticoid receptor activation

Sargis R., Johnson D., Choudhury R., Brady M., *Obesity* (2009) in press, doi:10.1038/oby.2009.419.

Rating 3b

Study objective:
To analyse *in vitro* the role of BPA via activation of the glucocorticoid receptor on cell adipogenesis in the 3T3-L1 cell line (preadipocytes).
Methods:
Study of the effects of various endocrine disruptors (BPA, DCHP (dicyclohexyl phthalate), endrin and TF (tolylfluanid)) at concentrations from 100 pmol/L to 1 µmol/L on glucocorticoid receptor activation in preadipocytes of the 3T3-L1 cell line in the presence of dehydrocorticosterone (DHC) required for the differentiation of preadipocytes into adipocytes.

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[5] Taylor J.A., Welshons W.V., vom Saal F.S. (2008). No effect of route of exposure (oral, subcutaneous injection) on plasma bisphenol A throughout 24 h after administration in neonatal female mice. *Reprod. Toxicol.* 25: 169-76.
[6] Lordosis: curvature of the spinal column.

3T3-L1 cells were transfected with a luciferase reporter containing a glucocorticoid response element. Lipid accumulation of differentiated 3T3-L1 adipocytes was determined by quantitative oil red O staining and immunoblotting for proteins induced during adipocyte differentiation (adiponectin, insulin receptor β, CCAAT/enhancer binding protein α).

Results:

- Of 13 selected endocrine disruptors, only 4, including BPA, activated the glucocorticoid receptor.
- At the concentration tested (100 nmol/L), the selected endocrine disruptors did not initiate adipogenesis when administered alone, but only when DHC was present. Under these conditions, the adipocyte-specific proteins were widely expressed, with BPA having less effect than the other endocrine disruptors.

The endocrine disruptors induced lipid accumulation in the adipocytes.

Study conclusion:

Endocrine disruptors can only promote differentiation of preadipocytes in synergy with other differentiation factors. This action involves the activation of the glucocorticoid receptor.

Comments of the BPA WG:

The conclusions of this article are not fully supported by the data obtained. The results showed that BPA supplemented the effect of the partial agonist (dehydrocorticosterone) of the glucocorticoid receptor (GR), but they did not show that this action was mediated by the GR, as the observed effects may also involve PPAR-γ. For example, no dose-response curve of BPA on the GR from 1 pM to 1 µM was shown. The use of a strong agonist of the GR allowed the authors to induce the expression of c/EBPα and adiponectin. For BPA, only one concentration (100 nM) seemed to induce adiponectin expression and no effect on c/EBPα was observed.

Moreover, the preadipocyte cell line no longer matched the original cell.

***In conclusion, the BPA WG** does not accept the results of this in vitro study as significant, whether with regard to BPA or the other substances tested.*

3.3.8 Perinatal exposure to bisphenol A alters early adipogenesis in the rat

Somm E., Schwitzgebel V.M., Toulotte A., Cederroth C.R., Combescure C., Nef S., Aubert M.L., Hüppi P., *Environmental Health Perspectives* (2009) 117:1549-1555.

Rating 3b

Study objective:

To determine whether perinatal exposure to BPA plays a role in adipogenesis of prepubertal animals.

Method and Results:

Female Sprague-Dawley rats had access to drinking water containing 1 mg/L of BPA from the 6th day of gestation until offspring were weaned on the 21st day (PND21), corresponding to an estimated exposure of 70 µg BPA/kg b.w./d.

- At birth, body weight, sex ratio and the number of offspring per litter were determined.
- At PND21, body weight and anogenital distance were measured. For some animals, periepididymal fat, parametrial fat, brown adipose tissue and liver were weighed and prepared for RNA quantification. The parametrial fat was studied by histology.

In the parametrial fat and liver of females, expression of the lipogenic genes (C/EBP-α, PPAR-γ SREBP-1C), lipogenic enzymes (LPL, FAS, SCD-1, ACC), lipogenesis inhibitors (GATA-2, Pref-1) and the glucose transporter (GLUT4) was determined. In blood, levels of glucose, cholesterol, triglyceride and non-esterified fatty acids were measured.

- For another group of animals, monitored from the 4th to the 14th week, half received standard feed and half a diet high in fat. The weight of the animals was monitored weekly. At the end of the study, the males were tested for glucose tolerance.

Results:

- At birth, treatment with BPA during gestation did not alter the sex ratio or litter size. However, treated infants of both sexes were heavier than the controls.
- At PND21, only the weight of treated females was increased. Parametrial fat was more abundant and displayed hypertrophied adipocytes. In these cells, lipogenic genes and those of lipogenic enzymes were overexpressed. In the liver, RNA levels of SREBP-1C, ACC and FAS were increased. Circulating lipids and glucose levels were normal.
- From 4-14 weeks, changes in body weight of BPA-treated males receiving the standard diet were similar to those of controls. On the other hand, males exposed to BPA and receiving the high-fat diet were heavier than the controls. In females, body weight was greater than that of controls for both diets tested. In males receiving the high-fat diet, the response to the glucose tolerance test was normal.

Study conclusion:
Perinatal exposure to BPA increases adipogenesis in females at weaning. In adult males, a weight increase is observed if the diet is rich in fat.
Comments of the BPA WG:
Cages and drinking bottles were made of polypropylene (no BPA), the drinking water of the controls contained negligible quantities of BPA and the food was low in phytoestrogens.
Nevertheless, this study had several methodological weaknesses: a limited number of litters (8), a single dose tested,; the experimental unit appeared to be the individual offspring and not the litter (even if an adjustment was made on litter size for the weight comparisons). The histology of parametrial adipose tissue should have been coupled with BrdU labelling to clarify the proliferative state of adipocytes.
This study suggested that perinatal exposure to BPA increased the sensitivity to weight gain, in a sex-dependent manner, even after weaning. Given the importance of the results, this would need to be confirmed through a study that is more robust from a methodological point of view.

***To conclude, the BPA WG** does not accept the results of this study as a warning signal, because of the methodological limitations.*

4. DATA RELATING TO EXPOSURE TO BISPHENOL A

4.1 Data relating to the release of BPA from polycarbonate baby bottles

BPA is authorised in the European Union for use in food contact materials with a specific migration limit (SML) of 0.6 mg/kg of food. In its initial 2006 Opinion on BPA, EFSA used a maximum concentration of 50 µg/L in beverages, particularly in infant formulae, for estimating exposure.

In its Opinion of 24 October 2008 regarding BPA in polycarbonate baby bottles likely to be heated in microwave ovens, AFSSA took into consideration data from Ehlert *et al.* (2008) and Kawamura *et al.* (1998) related to the migration of BPA from baby bottles containing distilled water and heated in a microwave oven under realistic conditions of either:

- three cycles of heating for three minutes (Ehlert *et al.,* 2008), or
- five minutes of heating (Kawamura *et al.,* 2008).

These studies reported values of the order of 0.1 to 0.7 µg/L of BPA leached into the water contained in the baby bottle. These values are well below the maximum concentration of 50 µg/L used by EFSA.

In addition, AFSSA emphasises that to reach the current TDI of 50 µg/kg b.w./d, a 3 month old infant weighing 6.1 kg would have to drink daily 1060 mL[7] of water or milk from a bottle leaching 287 µg of BPA per litre, a concentration that is practically six times higher than the value used by EFSA to estimate exposure.

AFSSA concluded that when the contents of polycarbonate baby bottles are heated in a microwave oven for less than ten minutes, the quantities of BPA that migrate into food are much less than the maximum value of 50 µg/L.
It was also noted that water hardness and detergent residues are factors that favour BPA migration from polycarbonate containers.

Given the recent publication of data on migration of BPA from polycarbonate containers (baby bottles, bottled water), AFSSA has conducted a review of the literature to assess the impact of these data on the conclusions of its Opinion of 24 October 2008.

- ***General remarks***

BPA that has leached into food from a polycarbonate (PC) container comes mainly from chemical degradation of the polymer rather than from migration (diffusion of residual monomers in the polymer and dissolution in the food). Its release depends on temperature, time, and the nature of the content tested (distilled water, tap water, food simulants). In worst-case conditions (high pH, hard water), the amount of BPA released may be higher than that accounted for in AFSSA's 2008 Opinion, but it should be noted that these test conditions were highly unlike the realistic conditions of use (the heating of milk in a baby bottle).

Some studies have compared different brands of baby bottles (without identifying them). The results show that the residual levels of BPA as a PC monomer vary widely (for example, Ehlert *et al.*, 2008), whereas concentrations of BPA in the media tested showed little variation. As mentioned above, this phenomenon is explained by the fact that BPA comes mainly from the degradation of the polymer (and not from the migration of residual monomers) and that the stability of the chemical bond of polycarbonate does not vary according to brand of baby bottle. For this reason, any differences between brands are not addressed in this report.

Studies conducted to estimate the quantities of BPA leached into beverages contained in PC baby bottles can be grouped into three categories:

1. Realistic conditions of use: heating the baby bottles in a water bath or by microwave (De Coensel *et al.,* 2009; Dutch report, 2005; Le *et al.,* 2008; Ehlert *et al.,* 2008; Kubwabo *et al.,* 2009; Maragou *et al.,* 2007).
2. Heating for prolonged periods, from 24 to 240 hours (Cao and Corriveau, 2008; Dutch report, 2005; Kubwato *et al.,* 2009; Maia *et al.,* 2009).

[7] Values used in EFSA's 2006 opinion, based on the German DONALD study (Kersting, 1998).

3. Heating to a very high temperature for home sterilisation (Brede *et al.,* 2003; Le *et al.,* 2008; Ehlert *et al.,* 2008; Maia *et al.,* 2009).

From these various conditions tested, it can be concluded that:

- At temperatures below 60°C, release of BPA into water is low and the final concentration is lower than 1 µg/L.
- Beyond this temperature (particularly for boiling water) the concentrations range from 14 to 137 µg/L (with a high variability according to pH).
- The maximum concentration of 137 µg/L corresponds to conditions that are highly unrealistic: the water that was used to sterilise the baby bottle (boiled, pH > 9) was used to fill the bottle, and was then heated for ten minutes to boiling.
- Detergents, particularly highly alkaline washing machine detergents, vigorously attack the PC especially at high temperatures, thus facilitating the release of BPA in the beverage that is then put into the baby bottle.

Based on these results and in order to minimise the release of BPA, the BPA WG recommends avoiding:

1. putting very hot beverages (>60 °C) in baby bottles,
2. using previously boiled water to reconstitute formula,
3. washing baby bottles with detergent at high temperatures (>50 °C), and
4. heating food (water, milk, soup, etc.) at very high temperatures in polycarbonate baby bottles or containers.

- ***Detailed review of the literature***

4.1.1 Release of bisphenol A from polycarbonate baby bottles: water hardness as the most relevant factor

Biedermann-Brem S. and Grob K., *European Food Research and Technology* (2009) 228: 679-684.

Rating 1

Study objective:
To study the effect of water hardness and pH on the release of BPA from polycarbonate baby bottles.
Method:
The baby bottles (n=2) were filled with 200 mL of water (1-ionised water, pH 5.0; 2-tap water, hardness[8] 35°f, pH 7.7; 3-drinking-fountain water, hardness 22°f, pH 7.4; 4- drinking-fountain water, hardness 37°f, pH 7.5) and heated in a microwave oven. The BPA concentration was measured by high performance liquid chromatography with fluorescence detection [HPLC-FLD], with a detection limit of 0.5 µg/L.
Results:
The release of BPA increased sharply with the pH of the water. Thus, when tap water was heated to boiling, evaporation of CO_2 caused an increase in pH to 9-9.5, which led to a chemical degradation of the PC and accelerated the release of BPA by a factor of 10 to 100. The worst case was observed with water used to sterilise the baby bottle in a microwave oven (boiled for several minutes) then reused to prepare the milk formula: the BPA concentrations observed were 36 µg/L after five minutes and 137 µg/L after ten minutes of heating, whereas, the concentration was only 1 µg/L after five minutes and 23 µg/L after ten minutes when unboiled water was used.
Comments of the BPA WG:
These test conditions were highly unlike realistic conditions of use.

***To conclude, the BPA WG** considers that these data cannot be used to estimate infant exposure.*

[8] Hardness is expressed in French degrees (°f): one °f equals 10^{-4} mol/L or 4 mg of calcium or 2.4 mg of magnesium per litre of water.

4.1.2 Release of bisphenol A from polycarbonate baby bottles: mechanisms of formation and investigation of worst case scenarios

Biedermann-Brem S., Grob K., Fjeldal P., *European Food Research and Technology* (2008) 227: 1053-1060.

Rating 2

Study objective:
To explain the results of Brede *et al.* (2003) that described concentrations of BPA increasing with the number of wash cycles.
Method:
'Worst case' scenarios of dishwasher washing were investigated. Indeed, dishwashing detergents are highly alkaline and lead to chemical attack on the polycarbonate. Baby bottles (four brands, one to two baby bottles/brand/test) were subjected to several tests.
- The residual content of BPA on the inside surface of the baby bottle was estimated by shaking 10 mL of methyl tertiary butyl ether (MTBE) in the baby bottle (the quantity considered to dissolve 1 mg of baby bottle or 50 nm thickness of PC). The results obtained with new baby bottles were compared with those obtained after 30 washing/drying cycles in the dishwasher.
- The effect of pH was studied with 100 mL of tap water (pH 6), a citric acid (pH 2) or sodium bicarbonate solution (pH 9) at 80°C for one hour.
- Improper positioning of the baby bottle in the dishwasher was simulated by placing a baby bottle containing 10 mL of a detergent solution or just tap water in a horizontal position (several brands were tested) then set on evaporation at 90°C for two hours.
The BPA concentration was measured using HPLC-FLD, with a detection limit of 0.01 µg/L.
Results:
The highest BPA concentrations were obtained when there was insufficient rinsing (due to machine malfunction or improper positioning) followed by drying at a high temperature. Nevertheless, under these conditions the observed values remained below the 50 µg/L used by EFSA.
Comments of the BPA WG:
This study shows that BPA is released by chemical attack (alkaline pH) and that the standardised migration tests are unable to simulate 'worst case' scenarios. Tests were carried out under a variety of conditions, but the exact number of baby bottles tested and the experimental protocols followed were not clearly described in the publication.
These test conditions were highly unlike realistic conditions of use.

***To conclude, the BPA WG** considers that these data cannot be used to estimate infant exposure.*

4.1.3 Increased migration levels of bisphenol A from polycarbonate baby bottles after dishwashing, boiling and brushing

Brede C., Fjeldal P., Skjevrak I., Herikstad H., *Food Additives and Contaminants* (2003) 20: 684-689.

Rating 1

Study objective:
To study the effects of dishwasher washing, boiling water, and the brushing of new or used PC baby bottles on the release of BPA.
Method:
Bottles made of PC (n=12) underwent repeated washing in the dishwasher (169 cycles) and were then tested, filled with boiling deionised water (200 mL), for one hour at 100°C.
The BPA concentration was measured by gas chromatography/mass spectrometry (GC-MS), with a detection limit of 0.1 µg/L.
Results:
The BPA concentrations observed in new baby bottles were around 0.2 µg/L; they increased with the number of wash cycles to 17 µg/L after 51 cycles and then decreased slightly after 169 cycles.
Comments of the BPA WG:
These test conditions were highly unlike realistic conditions of use.
At the time this study was published (2003), these results surprised the scientific community, since the presence of BPA had been explained up to that time by a phenomenon of migration that was assumed to decline with use. The studies conducted after this one showed that the source of BPA is in fact degradation of the PC and not migration of the residual monomer.

To conclude, the BPA WG *considers that these data cannot be used to estimate infant exposure. It also finds that the release of BPA comes mainly from the degradation of polycarbonate.*

4.1.4 Migration of bisphenol A from polycarbonate baby and water bottles into water under severe conditions

Cao X.-L. and Corriveau J., *Journal of Agricultural and Food Chemistry* (2008) 56: 6378-6381.

Rating 3

Study objective:
To measure the release of BPA from PC baby bottles and drinking bottles under severe conditions.
Method:
The release of BPA from PC baby bottles (n=3) and reusable drinking bottles (n=2) was measured by filling the containers completely with boiling water and then placing them in an oven at 70°C for one to six days.
The BPA concentration was measured by solid-phase microextraction (SPME) coupled with GC-MS, with a detection limit of 0.5 µg/L.
Results:
The BPA concentrations observed increased with contact time according to a second degree polynomial relationship (of the type $y = ax^2 + bx$, where y is the BPA concentration and x is the contact time), reaching values ranging between 61 and 105 µg/L after 48 hours.
Comments of the BPA WG:
The use of deionised water or tap water was not specified. Nevertheless, the authors mentioned the use of tap water for standard solutions, because the BPA concentration was too high in deionised water. Therefore, it seems likely that tap water was also used for testing. The pH of the water was 7, which corresponds to a pH before heating. The increase in the pH of the water by evaporation of CO_2 could explain the release of BPA.
These test conditions were highly unlike realistic conditions of use.

To conclude, the BPA WG *considers that these data cannot be used to estimate infant exposure.*

4.1.5 Study on the migration of bisphenol A from baby bottles by stir bar sorptive extraction-thermal desorption-capillary GC-MS

De Coensel N., David F., Sandra P., *Journal of Separation Science* (2009) 32: 1-8.

Rating 2

Study objective:
To study the release of BPA from PC baby bottles in conditions close to realistic conditions of use.
Method:
The tests were conducted with PC baby bottles (two brands, n=4, one baby bottle per temperature tested) filled with bottled water (200 mL) and heated to 37, 53, 65 or 86°C using microwave ovens of 500 W or 1000 W for 60 seconds or 20 to 90 seconds, respectively) and then left in contact with the water for 30 or 90 minutes. In another experiment, baby bottles were tested 100 times at different temperatures (n=4, one baby bottle per temperature tested). The effect of the heating method (by water bath or microwaves) was tested at 37-40°C and the influence of contact time was studied (0, 30 or 60 minutes). Glass baby bottles were used as controls.
The BPA concentration was measured using automated SPME coupled with GC-MS, with a detection limit of 0.12 ng/L.
Results:
The BPA concentration was 13 ng/L after 30 minutes at 37°C, 90 ng/L at 53°C and 1.5 µg/L at 86°C. No difference was observed between heating at 37-40°C by water bath and by microwave. No effect of the microwave oven power (1000 or 500 W; tested at 37°C), nor contact time (0 to 60 min) was demonstrated. The authors noted that all the tested waters (deionised, bottled) showed traces of BPA (average of bottled waters = 8 ± 1.5 ng/L). The release of BPA was greater during the first heating cycles (varying according to the temperature tested, from 15 to 296 ng/L, at 37 and 65°C respectively) then decreased to reach a constant value after 60 cycles (< 20 ng/L at 37°C and 226 ng/L at 65°C). It should be noted that after 20 heating cycles, no differences were found between the brands of baby bottles tested. Sterilisation (ten minutes in boiling water) increased the release of BPA by four to seven times (20 at 90 ng/L without and with sterilisation, respectively). The authors suggested that the BPA released during sterilisation was adsorbed on the walls of the baby bottle during drying.

Comments of the BPA WG:
The publication was based on a particularly sensitive method. The medium tested was bottled water. The experimental protocol was well described and robust: it is based on tests repeated 100 times and included glass baby bottles as controls.
These values were obtained in conditions close to realistic conditions of use.

***To conclude, the BPA WG** considers that these data can be used to estimate infant exposure.*

4.1.6 Migration of BPA and plasticizers from plastic feeding utensils for babies

Dutch Food and Consumer Product Safety Authority, Report no. ND05o410, June 2005

Rating 2

Study objective:
To verify that baby bottles and flexible plastic feeding utensils that are marketed in the Netherlands comply with regulations for food contact materials.
Method:
New (n=22, of 14 brands) and used baby bottles (n=20 of 11 brands, duration of use from three to 36 months) were tested at 40°C for 24 hours with 100 mL of simulants (distilled water or acetic acid 3%) to verify compliance for food contact.
The BPA concentration was measured by HPLC-FLD, with a detection limit of 2.5 µg/L in distilled water and 3.9 µg/L in acetic acid.
Results:
The maximum concentration measured was 5 µg/L in distilled water for four of the 20 used baby bottles. For the other used baby bottles and all of the new baby bottles, the values were below the detection limit. This study showed no difference according to brands or duration of use of the baby bottles.
Comments of the BPA WG:
This study showed the compliance of BPA tested under regulation testing conditions. The values obtained were well below the specific migration limit (SML) of 600 µg/L.
The test conditions (contact time of 24 hours) were highly unlike realistic conditions of use.

***To conclude, the BPA WG** considers that these data cannot be used to estimate infant exposure.*

4.1.7 Migration of bisphenol A into water from polycarbonate baby bottles during microwave heating

Ehlert K.A., Beumer C.W.E., Groot M.C.E., *Food Additives and Contaminants* (2008) 25: 904–910.

Rating 2

Study objective:
To measure the release of BPA from PC baby bottles after heating in a microwave oven.
Method:
Baby bottles made from PC (n=18, of 18 brands) were analysed for residual levels of BPA (as a monomer, by putting 10 mL of dichloromethane on a 1 g fragment of the baby bottle) and its release into distilled water (100 mL) after three heating cycles in a microwave oven at 100°C for three minutes. The baby bottles were tested twice.
The BPA concentration was determined by GC-MS, with a detection limit of 0.1 µg/L.
Results:
The mean concentrations measured in water were less than 1 µg/L (between 0.1 and 0.7 µg/L). The initial residual BPA content in the polycarbonate baby bottles ranged from 1.4 to 35.3 mg/kg. No correlation between the residual BPA level and concentration in water was shown, confirming that BPA comes from depolymerisation and not from migration. The authors also concluded that microwaves did not influence the release of BPA.
Comments of the BPA WG:
This study, sponsored by Plastics Europe, was conducted by an independent technology transfer center (TNO, Netherlands).

***To conclude, the BPA WG** considers that the data obtained under conditions consistent with conditions of use (3 minutes of heating in microwave ovens) can be used to estimate infant exposure.*

4.1.8 Migration of bisphenol A from plastic baby bottles, baby bottle liners and reusable polycarbonate drinking bottles

Kubwabo C., Kosarac I., Stewart B., Gauthier B.R., Lalonde K., Lalonde P.J., *Food Additives and Contaminants* (2009) 26: 928–937.

Rating 2

Study objective:
To measure the release of BPA from a variety of plastic containers including PC baby bottles, non-PC baby bottles, and reusable PC drinking bottles under conditions of repeated use.
Method:
Baby bottles made of PC (n=14, of nine brands) and plastics other than PC (n=10 including three made of polyether sulfone, five of polypropylene and two unknown), filled with water, 10% ethanol or 50% ethanol were maintained at 40°C in a water bath for 8, 24 or 240 h. Tests were also conducted by filling the baby bottles with boiling water or 10% ethanol at 85°C, then maintained at 60°C for 24 hours.
The effect of dishwasher washing was studied with PC baby bottles (one wash per day at 55°C for six days).
The BPA concentration was measured by gas chromatography/electron ionisation tandem mass spectrometry (GC-EI/MS/MS), with a detection limit of 0.04 ng/L.
Results:
The BPA concentrations measured from PC baby bottles increased from 0.11 µg/L in water at 40°C for eight hours, to 2.39 µg/L in 50% ethanol at 40°C for 240 hours. Residual BPA leaching from PC bottles increased with temperature (60°C) and contact time, the maximum value being 6.5 µg/L for heating at 60°C for 24 hours. Traces of BPA were shown for non-PC containers (<0.1 µg/L after ten days at 100°C). Repeated cleaning of the baby bottles in the dishwasher had no significant effect on the release of BPA. Concerning the reusable water bottles made from PC, the concentrations measured ranged from 0.01 to 2.16 µg/L (40°C for 2 to 240 hours).
Comments of the BPA WG:
These results are consistent with those already published in the literature.

***To conclude, the BPA WG** considers that the data obtained in conditions close to realistic conditions of use (tests at 40°C) can be used to estimate infant exposure.*

4.1.9 Effect of detergents in the release of bisphenol A from polycarbonate baby bottles

Maia J., Cruz J.M., Sendón R., Bustos J., Sanchez J.J., Paseiro P., *Food Research International* (2009) 42: 1410–1414.

Rating 1

Study objective:
To study the effect of various types of detergents on the release of BPA from PC baby bottles.
Method:
Five different detergents and bleach were tested to assess their impact on the release of BPA from polycarbonate baby bottles. The commercial baby bottles (unspecified number of samples) were cut into pieces measuring 10 cm^2. The tests were conducted by placing three pieces (or 30 cm^2) in 15 mL of aqueous solution with 1% detergent for one hour at 120°C, then 30 minutes at room temperature and finally 120 hours (five days) at 25°C. After rinsing with distilled water, the pieces of polycarbonate were exposed for three one-hour cycles at 120°C, followed by 30 minutes at room temperature.
The BPA concentration was measured by HPLC-FLD, with a detection limit of 5 µg/L.
Results:
The release of BPA in the washing solutions after one hour at 120°C ranged from 22 to 54,800 µg/L, the minimum value being observed for one of the two hand dishwashing detergents and the maximum value for the other (probably with a neutral pH). Dishwasher detergents induced the release of BPA ranging from 809 to 30,900 µg/L. The release of BPA remained high after rinsing with distilled water following incubation for one hour at 120°C. It should be noted that in the control case, conducted with distilled water only, the release of BPA was 109 µg/L after one hour at 120°C. Bleach did not induce the release of BPA (the measured level was below the detection limit in the first cycle and 45 µg/L in the second one-hour cycle at 120°C).
Comments of the BPA WG:
The BPA concentrations observed are explained by the high temperature (120°C), by a larger polycarbonate/liquid ratio than in real conditions (2 cm^2/mL instead of approximately 0.8 cm^2/mL) and

by the highly alkaline pH of the dishwasher detergents. However, the authors give no explanation and provide no information concerning the composition or the pH of these detergents.
The BPA WG noted the value of these studies on the use of hand dishwashing *detergents (pH neutral, but no information was given on their identity or composition), which led to a high release in the washing solution, as well as in distilled water heated in the baby bottles after rinsing (simulating a beverage consumed in a washed baby bottle). The study conditions were not representative of conditions of use (1 h/120°C), but after adjusting the 'baby bottle surface area to beverage volume' ratio, the release would be 320 times above the limit of 50 µg/L. If these results are confirmed by other studies, they could indicate a situation leading to a release of BPA higher than that expected to date.*
These test conditions were highly unlike realistic conditions of use.

***To conclude, the BPA WG** considers that these data cannot be used to estimate infant exposure.*

4.1.10 Migration of bisphenol A from polycarbonate baby bottles under real use conditions

Maragou N.C., Makri A., Lampi E.N., Thomaidis N.S., Koupparis M.A., *Food Additives and Contaminants* (2008) 25: 373–383.

Rating 2

Study objective:
To study the release of BPA from PC baby bottles in conditions close to realistic conditions of use: effects of sterilisation in boiling water, washing in a dishwasher or with a brush, and of temperature.
Method:
The release of BPA from PC baby bottles (n=31, of six brands) was studied with aqueous simulants (water or acetic acid 3%) under repetitive use. The tests were conducted with heating at 70°C for two hours or by filling with boiling water followed by contact for 45 min at room temperature. Temperature was the main factor studied. The experiments were conducted by repeated cycles of washing at 60°C, rinsing with distilled water, sterilisation for ten minutes in boiling water, then a test of migration into the simulant at 70°C for two hours.
The BPA concentration was measured by HPLC-FLD, with a detection limit of 2.5 µg/L and 1.8 µg/L in water and acetic acid respectively.
Results:
During the first 15 cycles, in tests conducted at 70°C, the BPA concentration in the water was below the detection limit. The BPA concentrations were measurable only after the bottles were filled with boiling water (condition tested starting from the 16th cycle), with a maximum value of 14.3 µg/L. Neither the acidity of the simulant (to simulate fruit juice) nor washing with manual brushing increased the BPA concentration. The study of the release of BPA with boiling water showed an increase during the first four to eight cycles and then a decrease up to the 12th cycle of use. The authors observed a decrease in the release of BPA with the number of ten-minute sterilisation cycles in boiling water (in contrast with the study of Brede *et al.*, 2003). They concluded that residual BPA should be present on the inside surfaces of PC baby bottles and that polymer degradation did not occur.
Comments of the BPA WG:
These results are consistent with those already published. The tests conducted at 70°C for two hours did not detect BPA in the water. Only the tests conducted with baby bottles filled with boiling water and then left in contact for 45 minutes, led to a release of BPA ranging from 2.4 to 14.3 µg/L.
These test conditions were highly unlike realistic conditions of use and the detection limits were too high for the results of tests conducted at 70°C to be taken into account.

***To conclude, the BPA WG** considers that these data cannot be used to estimate infant exposure.*

4.1.11 Conclusions of the BPA WG

To estimate dietary exposure to BPA in infants (see paragraph 4.3), the BPA WG used three studies conducted in conditions similar to realistic conditions of use of baby bottles. These are the studies by Ehlert *et al.,* 2008 (three times three minutes at 100°C), De Coensel *et al.*, 2009 (30 minutes at 37°C) and Kubwabo *et al.*, 2009 (eight hours at 40°C). The BPA concentration ranged from < 0.1 to 0.7 µg/L.

4.2 Data relating to the release of BPA from other containers for babies

4.2.1 Levels of bisphenol A in canned liquid infant formula products in Canada and dietary intake estimates

Cao XL, Dufresne G, Belisle S, Clement G, Falicki M, Beraldin F, Rulibikiye A., *Journal of Agricultural and Food Chemistry* (2008) 56: 7919-7924.

Rating 2

Study objective:
To measure the BPA concentration in liquid infant formula contained in metal cans marketed in Canada, in order to estimate daily intake of BPA.
Method:
The BPA concentration was measured by GC-MS in 21 samples, with a detection limit of 0.5 ng/g.
Results:
BPA was detected in all the samples at concentrations ranging from 2.27 ng/g to 10.2 ng/g. The daily intake for babies aged 0-1 month was estimated at 1.35 µg/kg b.w./d.
Comments of the BPA WG:
This study confirmed the presence of BPA in liquid infant formula by migration from resins used in the internal coating for food and beverage cans. These concentrations were slightly lower than those typically observed in canned products.

***To conclude, the BPA WG** considers that these data can be used to estimate exposure of infants and young children to BPA from infant formula.*

4.2.2 Levels of bisphenol A in canned soft drink products in Canadian markets

Cao XL, Corriveau J, Popovic S., *Journal of Agricultural and Food Chemistry* (2009) 57:1307-1311.

Rating 2

Study objective:
To measure the release of bisphenol A in soft drink cans stored at room temperature and marketed in Canada.
Method:
The BPA concentration was measured by GC-MS in 72 canned soft drink products with a detection limit of 0.045 µg/L.
Results:
The BPA concentrations ranged from 0.03 to 4.5 µg/L. About 75% of the products had BPA levels less than 0.5 µg/L, and 85% less than 1 µg/L. On the basis of the consumption of one canned soft drink with the highest BPA level (4.5 µg/L) , the daily intake for one 60 kg adult was estimated by the authors at 0.027 µg/kg b.w./d.
Comments of the BPA WG:
This study showed the presence of BPA in canned beverages, by migration from resins used in the internal coating of cans.

***To conclude, the BPA WG** considers that these data can be used to estimate adult and infant exposure to BPA through the consumption of canned beverages.*

4.2.3 Bisphenol A in baby food products in glass jars with metal lids from Canadian markets

Cao XL, Corriveau J, Popovic S, Clement G, Beraldin F, Dufresne G., *Journal of Agricultural and Food Chemistry* (2009) 57: 5345-5351

Rating 2

Study objective:
To measure the BPA concentration in baby food products contained in small glass jars with plastic film-coated metal lids.
Method:
The BPA concentration was measured in 99 baby food products from seven suppliers, by GC-MS with a detection limit of 0.18 ng/g.

Results:
Of these 99 products, 15 had levels below the detection limit (0.18 ng/g) and 70 less than 1 ng/g. The average was 1.1 ng/g and the maximum value was 7.2 ng/g. The BPA content in jars of fruit (0.6 ng/g) was lower than that of vegetables (1.2 ng/g).
Comments of the BPA WG:
This study showed that plastic film-coated metal lids can release BPA in food contained in small glass jars.

***To conclude, the BPA WG** considers that these data can be used to estimate the exposure of infants and young children to BPA through the consumption of food products in glass jars with metal lids.*

4.2.4 Migration of bisphenol A from can coatings to liquid infant formula during storage at room temperature

Cao XL, Corriveau J, Popovic S., *Journal of Food Protection* (2009) 72: 2571-2574.

Rating 1

Study objective:
To measure the BPA level in liquid infant formula (soy milk or cow's milk) contained in cans after storage at room temperature.
Method:
The BPA concentration was measured in two batches of 21 liquid infant formula samples (four soy-based products and 17 cow's milk-based products) contained in cans. The first batch was analysed in 2007 and the second ten months later, at a time close to their expiration dates, by GC-MS with a detection limit of 0.5 ng/g.
Results:
For 13 of the 21 samples, an increase of at least 10% in the BPA concentration was observed after ten months of storage. The values were between 2.3 and 10 µg/L in 2007 and 2.8 and 12 µg/L ten months later. A decrease of at least 10% was observed in one of the 21 samples. In the other cases, the variation was less than 10%.
For cow's milk, the BPA concentration was proportional to contact time (duration of storage), with a difference between manufacturers. The average BPA concentration was 5 ng/g in 2007 and 6.8 ng/g in 2008.
For soy milk, there was no significant difference (5.8 and 5.3 ng/g respectively).
Comments of the BPA WG:
This study showed that the effect of storage at room temperature was very limited.

***To conclude, the BPA WG** considers that these data can be used to estimate the exposure of infants and young children to BPA from liquid infant formula (even though the authors specified that 16 of the 21 samples were past the expiration date).*

4.2.5 Investigation of storage time on potential bisphenol A migration into canned liquid infant formula stored at room temperature - summary

Health Canada, December 2009, http://www.hc-sc.gc.ca/fn-an/pubs/securit/bpa-temp-eng.php

Rating 1

Study objective:
To look for possible changes in BPA concentrations in canned liquid infant formula products marketed in Canada, after extended storage (ten months) at room temperature.
Method:
The BPA concentration was measured in the remaining unopened cans (all from the same batch for each product) of the 21 canned liquid infant formula products (4 soy-based products and 17 cow's milk-based products) that were analysed for a previous survey. Two subsamples from each sample were analysed by GC-MS with an average detection limit of 0.5 ng/g.
Results:
In 2007, BPA concentrations ranged from 1.14 to 5.44 ng/g with an overall average of 2.88 ng/g. After storage at room temperature for ten months, BPA levels ranged from 1.39 to 6.18 ng/g with an overall average concentration of 3.64 ng/g in the same products from the same batches.
When compared to the same products analysed ten months earlier, both increases and decreases were noted. Only 9 of 21 products had levels higher than that which could be attributed to within-batch

variations and repeatability of analysis. These findings indicate the possibility of BPA migration from can linings during extended storage at room temperature. It should be noted that 16 of 21 products were past the expiration date by 10 to 206 days when they were analysed. However, no obvious correlation between the product expiration date and the level of BPA migration was found.
The variation in BPA concentration among different canned liquid infant formula products could be due to the differences in can linings or sterilisation methods (temperature and duration) used by different infant formula manufacturers. There is also evidence that the contents of the can may affect migration of BPA.
Comments of the BPA WG:
As in the work of Cao et al. (2009), this study showed that the effect of storage at room temperature on the release of BPA is very limited. However, the BPA WG questions the source of discrepancies between the values observed in these two studies that appeared to use similar experimental protocols.

***To conclude, the BPA WG** considers that these data can be used to estimate the exposure of infants and young children to BPA through the consumption of liquid infant formula.*

4.2.6 Survey of Bisphenol A in canned liquid infant formula products

Health Canada, August 2008, http://www.hc-sc.gc.ca/fn-an/securit/packag-emball/bpa/bpa_survey-enquete-eng.php

Rating 2

Study objective:
To measure BPA concentration in canned products intended for babies, marketed in Canada.
Method:
BPA concentration was measured in 21 liquid infant formula products of various brands. These products were marketed under 8 brands by 4 different companies. Among the 21 products, 17 were milk-based and 4 were soy-based; 18 were concentrated and 3 were ready-to-use products. The results were adjusted to account for the recommended dilution factor for the concentrated products. All the samples were stored at room temperature before analysis. Two subsamples from each sample were analysed by GC-MS with an average detection limit of 0.5 ng/g.
Results:
BPA concentrations ranged from 1.14 to 5.44 ng/g with an average of 2.88 ng/g.
Comments of the BPA WG:
This study confirmed the presence of BPA in liquid infant formula, by migration from resins used in the internal coating of cans.

***To conclude, the BPA WG** considers that these data can be used to estimate the exposure of infants and young children to BPA through the consumption of liquid infant formula.*

4.2.7 Survey of bisphenol A in canned powdered infant formula products

Health Canada, July 2009, http://www.hc-sc.gc.ca/fn-an/pubs/securit/bpa_survey-enquete-pow-pou-eng.php

Rating 2

Study objective:
To measure the BPA concentration in canned powdered infant formula products, marketed in Canada.
Method:
The BPA concentration was measured in 38 canned powdered infant formula products that were marketed under 11 brands by six different companies. Among the 38 products, 31 were milk-based and seven were soy-based. The samples were analysed by GC-MS with an average detection limit of 0.13 ng/g.
Results:
The BPA concentrations were below the detection limit in the 38 products analysed.
Comments of the BPA WG:
This study confirmed the presence of BPA in canned powdered infant formula products, by migration from resins used in the internal coating of cans.

***To conclude, the BPA WG** considers that these data can be used to estimate the exposure of infants and young children to BPA through the consumption of powdered infant formula.*

4.2.8 Survey of bisphenol A in baby foods prepackaged in glass jars with metal lids

Health Canada, July 2009, http://www.hc-sc.gc.ca/fn-an/pubs/securit/bpa_survey-enquete-eng.php

Rating 2

Study objective:

To measure the BPA concentration in baby foods prepackaged in glass jars with metal lids.

Method:

The BPA concentration was measured in 122 baby food products prepackaged in glass jars with metal lids that were marketed under 7 brands by 6 different companies. The presence of BPA could not be quantified for 23 products due to interference from sample food matrices. Among these products, 14 products were classified as mixed dishes, 7 products were classified as fruit-based, 1 product was classified as vegetable-based and 1 product was a beverage.

Of the 99 products where BPA could be quantified, 34 were classified as vegetable-based, 31 products were classified as mixed dishes, 29 products were classified as fruit-based, 3 products were classified as a dessert, 1 product was classified as a cereal and 1 product was a beverage. These products covered at least 80% of the market share of baby food products sold in Canada. Two subsamples from each sample were analysed by GC-MS with an average detection limit of 0.18 ng/g.

Results:

In 15% of the products, the BPA concentration was below the average detection limit. Approximately 70% of the products had BPA levels less than 1.0 ng/g. The average BPA concentration for all products was 0.95 ng/g. Variations of BPA levels in different baby food products (0.19 to 7.22 ng/g) could be due to the differences in metal lid coatings or sterilisation conditions (temperature and duration) used for different baby food products.

Comments of the BPA WG:

This study showed that plastic film-coated metal lids can release BPA in foods contained in small glass jars.

***To conclude, the BPA WG** considers that these data can be used to estimate the exposure of infants and young children to BPA through the consumption of food products in glass jars with metal lids.*

4.2.9 Conclusions of the BPA WG

In order to estimate infant dietary exposure to BPA (see paragraph 4.3), the BPA WG used two studies that measured the presence of BPA in liquid infant formula. In the study by Cao *et al.* 2009 (21 samples), the average from the analyses conducted 10 months apart was 5 and 6.8 ng [BPA]/g and the maximum value was 12 ng [BPA]/g.

In the 2008 Health Canada study (21 samples analysed), the average was 2.88 ng [BPA]/g, with a maximum value of 5.44 ng [BPA]/g and a minimum value of 1.14 ng [BPA]/g.

4.3 Estimated infant dietary exposure to BPA

Infant dietary exposure to BPAwas estimated on the basis of daily milk consumption of 174 mL/kg of body weight (Kersting *et al*., 1998)[9]. Three sources of exposure were taken into account: polycarbonate (PC) baby bottles , infant formula, and breast milk.

Exposure from PC baby bottles
According to studies by Ehlert *et al.,* (2008), De Coensel *et al*. (2009) and Kubwabo *et al*. (2009), infant exposure was estimated to be between 0.017 and 0.12 µg/kg b.w./d.

Exposure through infant formula
According to studies by Cao *et al.* (2009) and Health Canada (2008), infant exposure was estimated to be between 0.20 and 2.1 µg/kg b.w./d.

Exposure through breastfeeding[10]
The BPA WG used two studies that measured the presence of BPA in breast milk. In the study by Sun *et al*., 2004 (23 samples obtained from Japanese women), the average was 0.61 µg [free BPA]/L and the maximum value was 0.97 µg [free BPA]/L. In the study by Ye *et al*., 2006 (20 samples obtained from American women), the average and maximum values were 1.3 and 6.3 µg [free BPA]/L and 1.9 µg and 7.3 µg [total BPA]/L, respectively.
According to these two total BPA values in breast milk, infant exposure was estimated to be between 0.33 and 1.27 µg/kg b.w./d.

The following table shows a summary of estimated infant dietary exposure to BPA, according to the scenario used.

Table 2: Estimated infant dietary exposure to BPA, according to the scenarios used for daily consumption of 174 mL/kg b.w.

Source of exposure	Maximum value	Average	Minimum value
Polycarbonate baby bottles *Estimated exposure*	0.7 ng/mL *0.12 µg/kg b.w./d*	0.11 ng/mL *0.019 µg/kg b.w./d*	0.1 ng/mL *0.017 µg/kg b.w./d*
Infant formula *Estimated exposure*	12 ng/g *2.1 µg/kg b.w./d*	2.88 ng/g *0.50 µg/kg b.w./d*	1.14 ng/g *0.20 µg/kg b.w./d*
Total estimated exposure	*2.2 µg/kg b.w./d*	*0.52 µg/kg b.w./d*	*0.22 µg/kg b.w./d*
Breast milk *Estimated exposure*	7.3 µg/L *1.27 µg/kg b.w./d*	1.9 µg/L *0.33 µg/kg b.w./d*	

To refine these estimates of infant dietary exposure, the BPA WG recommends acquiring French data on BPA in breast milk and infant formula.

[9] Kersting, M., Alexy, U., Sichert_Hellert, W., Manz, F. and Schoch, G. (1998). Measured consumption of commercial infant food products in German infants: results from the DONALD study (DOrtmund Nutritional and Anthropometrical Longitudinally Designed). *J. Pediatr. Gastroeneterol. Nutr.* 27: 547-552.

[10] Sun, Y., Irie, M., Kishikawa, N., Wada, M., Kuroda, N., and Nakashima, K. (2004). Determination of bisphenol A in human breast milk by HPLC with column-switching and fluorescence detection. *Biomed Chromatogr* 18, 501-7.
Ye X., Kuklenyik Z., Needham L.L., Calafat A.M. (2006). Measuring environmental phenols and chlorinated organic chemicals in breast milk using automated on-line column-switching-high performance liquid chromatography-isotope dilution tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 831(1-2):110-5.

4.4 Data relating to the release of BPA from cans and polycarbonate bottles

Concerning populations other than infants (adults and children with a varied diet), other sources of dietary exposure to bisphenol A should be considered, such as canned foods and beverages, plastic containers, etc.

4.4.1 Dietary exposure assessment of pregnant women to bisphenol A from cans and microwave containers in Southern Spain

Mariscal-Arcas M., Rivas A., Granada A., Monteagudo C., Murcia M.A., Olea-Serrano F., *Food and Chemical Toxicology* (2009) 47: 506-510.

Rating 2

Study objective:
To measure the BPA concentration in cans and containers that may be heated in a microwave oven to estimate dietary exposure of pregnant women in Spain.
Method:
The study examined a cohort of mother-son pairs established at Granada University Hospital (2000-2002). The day after delivery, women answered a frequency questionnaire on food consumption. The number of individuals used in the study was not specified. The data on BPA contamination used to calculate exposure were not BPA concentrations in food, but the amount of BPA released from containers that were emptied, filled with deionised water and autoclaved for 30 minutes at 125°C. The microwave containers were tested with deionised water heated for one minute. Analyses were conducted by HPLC-FLD with a limit of quantification of 22.8 ng/mL and included 45 canned products (20 vegetables, 10 pulses, 5 meats, and 10 fish), 20 canned beverages and 20 plastic containers.
Results:
The average levels of BPA ranged from 1.3 µg/L for microwavable containers to 25 µg/L for canned pulses (the maximum level was 54 µg/L for canned vegetables and canned pulses). Based on these results, the exposure of pregnant women in Spain was estimated at 1.1 µg/d.
Comments of the BPA WG:
This study shows the migration of BPA from resins used in the internal coating of cans. and from plastic containers heated in a microwave oven.

***To conclude, the BPA WG** considers that these data can be used to estimate dietary exposure to BPA linked to consumption of canned goods and the use of plastic containers heated in a microwave oven.*

4.4.2 Bisphenol A is released from polycarbonate drinking bottles and mimics the neurotoxic actions of estrogen in developing cerebellar neurons

Le H.H., Carlson E.M., Chua J.P., Belcher S.M., *Toxicology Letters* (2008) 176: 149–156.

Rating 2

Study objective:
To measure the release of BPA from new and used PC bottles, and study its estrogen-mimicking activity and neurotoxicity in developing cerebellar neurons.
Method (concerning the measurement of BPA release only):
The release of BPA in distilled water (100 mL) from new (n=3) and used (n=5) PC bottles as well as high-density polyethylene bottles (HDPE, n=3) was measured at room temperature (22°C) for 1, 3, 5 and 7 days. At 100°C, two new PC bottles and one used PC bottle were tested for 24 hours. The tests were conducted on three subsamples from each sample.
The BPA assay was performed by an immunochemical method (ELISA) with a detection limit ranging from 0.05 to 10 µg/L depending upon the tests.
Results:
The release of BPA from used PC bottles was below 1 µg/L (0.21 to 0.93 µg/L) after 1 to 7 days at room temperature and below 2 µg/L after 24 hours at 100°C. For new bottles, the release of BPA was below 1 µg/L (0.08 to 0.36 µg/L) after the first day, then increased between 0.7 and 1.33 µg/L after 7 days at room temperature. At 100°C, the values remained below 8 µg/L after 24 hours (3.84 to 7.67 µg/L). The release of BPA thus increased with time and water temperature (it was 15 to 55 times higher at 100°C than at 22°C). Concerning the HDPE bottles, BPA concentrations varied from < detection limit to 0.19 µg/L.

Comments of the BPA WG:
The values are consistent with those published in the literature.

***To conclude, the BPA WG** considers that these data cannot be used to estimate the exposure of the French population, because these types of PC water bottle are not marketed in France.*

4.4.3 Determination of bisphenol A in canned fatty foods by coacervative microextraction, liquid chromatography and fluorimetry

Perez-Bendito M.D., Bravo S.R., Lunar Reyes M.L., Garcıa Prieto A., Food Additives and Contaminants 26 (2009), 265–274.

Rating 2

Study objective:
To measure BPA concentrations in cans marketed in Spain.
Method:
The BPA concentration in canned fish or shellfish (n=4, tuna/mackerel/sardines in olive oil, mussels in pickled sauce) and meat products (n=2, meatballs/cooked pork) was measured by HPLC-FLD with a limit of quantification of 15 to 29 ng/g.
Results:
The BPA concentration in canned fish or shellfish products ranged from 20 to 129 ng/g and that of the meat was non-detectable (< 9 ng/g) to 37 ng/g.
Comments of the BPA WG:
The values are consistent with those published in the literature.

***To conclude, the BPA WG** considers that these data can be used to estimate dietary exposure to BPA linked to the consumption of canned foods.*

4.4.4 Survey of bisphenol A in bottled water products

Health Canada, July 2009, http://www.hc-sc.gc.ca/fn-an/securit/packag-emball/bpa/bpa_survey-enquete-bot-bou-eng.php

Rating 1

Study objective:
To measure the BPA concentration in bottled water marketed in Canada.
Method:
The BPA concentration was measured in 54 bottled water products marketed under 21 brands by 16 different companies, covering spring, mineral, flavoured and non-flavoured, carbonated and non-carbonated waters. The containers were made of glass, metal, high density polyethylene (HDPE), polyethylene terephthalate (PET) and polycarbonate (PC). Containers for all PC bottled water products were 18.5 litre carboys.
A total of 68 samples from 54 bottled water products were collected. Two subsamples from each sample were analysed by GC-MS with an average detection limit of 0.5 µg/L.
Results:
The BPA concentration in samples from all 51 non-polycarbonate bottled water products were below the detection limit. BPA was detected in 13 of the 17 samples from 4 of the 5 waters bottled in polycarbonate containers. The concentrations of BPA ranged from 0.50 to 8.82 µg/L, with an average of 1.5 µg/L.
Comments of the BPA WG:
The values are consistent with those published in the literature.

***To conclude, the BPA WG** considers that these data can be used to estimate BPA exposure through the consumption of bottled water.*

4.4.5 Survey of bisphenol A in canned drink products

Health Canada, March 2009, http://www.hc-sc.gc.ca/fn-an/securit/packag-emball/bpa/bpa_survey-enquete-can-eng.php

Rating 1

Study objective:
To measure the BPA concentration in canned drink products marketed in Canada.
Method:
The BPA concentration was measured in 72 canned drink products of different types including diet, non-diet, fruit-flavoured drinks, energy drinks, and other varieties. These products represented at least 84% of the market share of soft drink products sold in Canada. All of these products were carbonated except for four tea-based drinks.
Two subsamples from each sample were analysed by GC-MS with an average detection limit of 0.045 µg/L.
Results:
Due to the sensitivity of the method used, BPA was detected in all drink products, except for two tonic water soda products and one energy drink product. It appears that quinine hydrochloride, which is commonly used as a bittering agent in tonic type drinks, may interfere with BPA extraction.
In 75% of the products, the BPA concentration was below the detection limit; 85% of the products had less than 1 µg/L. The average contamination in all products was established at 0.57 µg/L. Differences in can linings or can sterilisation conditions (temperature and duration) used by different canned drink product companies could explain the variation of BPA concentration in different canned drink products (0.032 to 4.5 µg/L). Accidental exposure of the canned drink products to heat (e.g. sunlight) during storage or transportation could also be a factor that potentially increases BPA migration to the beverage.
Comments of the BPA WG:
The values are consistent with those published in the literature.

***To conclude, the BPA WG** estimates that these data can be used to estimate BPA exposure through the consumption of canned beverages.*

4.5 Data relating to non-food sources of exposure to BPA

Finally, other non-dietary sources of exposure to BPA should be investigated, as suggested by the following twopublications.

4.5.1 Assessment of human exposure to bisphenol A, triclosan and tetrabromobisphenol A through indoor dust intake in Belgium

Geens T., Roosens L., Neels H., Covaci A., *Chemosphere* (2009) 76: 755–760.

Rating 1

This study concerns the analysis by liquid chromatography – tandem mass spectrometry (LC-MS/MS) of BPA, tetrabromobisphenol A (TBBPA) and triclosan residues in indoor dust samples from 18 homes and two offices in Flanders, Belgium. The median values reported for BPA were 1500 ng/g in the homes and 6500 ng/g for the two offices. The values observed in the homes were 3 times higher than those previously reported in the United States (Rudel *et al.,* 2003) or in Germany (Volkel *et al*., 2008). These differences were not explained. Based on the calculations of Lakind and Naiman (2008) (extrapolated from urinary concentrations) of a daily exposure (all routes combined) of 3500 ng for a 70 kg adult, the authors suggested that inhalation is a minor route of exposure to BPA, but did not establish proof.
Comments of the BPA WG:
The authors did not provide any information or hypothesis to explain the origin of BPA in household dust. These data should be verified.
In addition, the previously-published data on the presence of BPA in indoor dust and outside air are shown in Table 3.

Table 3: Data relating to the presence of BPA in indoor dust and outside air.

Country	Matrix	BPA concentrations	Reference
USA	Office and household dust	0.25 – 0.48 µg/g	Rudel *et al.*, 2001*
USA	Workplace and residential indoor air	< 0.1 – 1.80 ng/m³	Wilson *et al.*, 2001*
	Outside air	< 0.1 – 2.50 ng/m³	
	Classroom floor dust	1.04 – 4.51 µg/g	
USA	Air inside the home	< 0.1 – 29 ng/m³	Wilson *et al.*, 2003*
	Air inside the nursery	2.81 – 8.80 ng/m³	
	Air outside the home	< 0.1 – 4.41 ng/m³	
	Air outside the nursery	0.16 – 4.72 ng/m³	
	Household dust	0.707 – 1.89 µg/g	
	Nursery dust	0.57 – 3.26 µg/g	
USA	Household dust	Median: 820 ng/g	Rudel *et al.*, 2003
Germany	Aerosols	5 – 15 pg/m3	Berkner *et al.*, 2004*
Japan	Urban air	< 0.1 – 1.92 ng/m³	Matsumoto *et al.*, 2005*
USA	Air inside the home	< 0.09 - 193 ng/m³	Wilson *et al.*, 2007
	Air inside the nursery	< 0.09 – 8.99 ng/m³	
	Air outside the home	< 0.09 – 44.6 ng/m³	
	Air outside the nursery	< 0.09 – 51.5 ng/m³	
	Household dust	< 2 – 707 ng/g	
	Nursery dust	< 2 – 156 ng/g	
Germany	Household dust	Median: 555 ng/g	Volkel *et al.*, 2008
Belgium	Household dust	535 – 9730 ng/g	Geens *et al*, 2009
	Office dust	4685 – 8380 ng/g	

** in Tsai et al. (2006)*

***To conclude, the BPA WG** considers that studies should be conducted to confirm or refute the presence of BPA in household dust in France.*

4.5.2 Exposure analysis of bisphenol A in surface water systems in North America and Europe

Klecka G.M., Staples C.A., Clark K.E., van der Hoeven N., Thomas D.E., Hentges S.G., *Environmental Science and Technology* (2009) 43: 6145–6150.

Rating 1

This article is a review of 89 studies investigating BPA in fresh surface waters between 1997 and 2007. Based on 1068 and 848 analyses of aquatic environments in North America and Europe respectively, BPA was detected in 20-51% of the samples. Median BPA concentrations were 0.081 µg/L in North America and 0.01 µg/L in Europe; the 95th percentiles were respectively 0.47 and 0.35 µg/L.

Comments of the BPA WG:

The BPA WG emphasises that these data concern only fresh surface water and do not reflect the impact of drinking water treatments.

The presence of BPA in surface waters and drinking waters intended for human consumption has already been addressed in this report (refer to page 6).

Moreover, at the request of the French Directorate General for Health, AFSSA's Laboratory for study and research on hydrology (LERH) plans to conduct a national campaign in 2010 to analyse BPA in raw water and in drinking water. Sampling should include 250 pairs of raw water/treated water samples, (500 samples in total). The objective is to measure the presence of BPA in the resources, the effectiveness of treatment processes and the possible contribution of materials used in the distribution networks. Results should be available in the first half of 2011.

***To conclude, the BPA WG** considers that the data of Klecka et al. (2009) cannot be used to estimate human exposure by the consumption of drinking water.*

4.5.3 Other non-dietary sources of exposure to BPA

In addition to the two potential sources of exposure to BPA described above, other sources should be considered, such as:

- Recycled paper and cardboard, due to the presence of BPA in thermal printer paper (faxes and cash register receipts[11], RAR 2010). The possible transfer of BPA from cardboard to foodstuffs has not been established and should be investigated. Human exposure resulting from skin contact with thermal printer paper should also be determined, especially since metabolism in this way may differ from that by oral administration.
- PVC plastic materials that may contain BPA.
- Dental sealants.

In order to reduce exposure to bisphenol A in the population, and, in particular, in pregnant women and young children, through the use of substitute products, the BPA WG stresses the importance of rigorous risk assessment processes for any product being considered as a substitute for bisphenol A. This assessment process is part of the European regulations, for which a reassessment of the previously approved monomers in light of current scientific knowledge would be relevant.

For example, polyether sulfone (PES) used to replace PC in baby bottles is a plastic material with bisphenol S as a monomer, whose toxicity is less understood that that of bisphenol A (the European assessment from 2000 was based on only four genotoxicity studies[12] and did not include any long-term study conducted on laboratory animals).

4.5.4 Other estrogen-mimicking compounds in food

Potential health risks linked to human exposure to BPA should be evaluated within the broader context of exposure to estrogen-mimicking compounds in food, whether naturally occurring compounds such as phytoestrogens or synthetic compounds such as nonylphenols.

Thus, dairy products are the main source of dietary steroids and total intake for an adult is estimated by Hartmann *et al.* (1998) to be 80-100 ng per day (or approximately 2 ng/kg b.w./d). Estradiol concentrations measured in milk indicate values of about 20 ng/L (Courant *et al.*, 2007). Measurement by bioassay of estrogenic potential (expressed as estradiol equivalent [EEQ]) would be 20 to 40 ng EEQ/L in beer (Promberger *et al.*, 2001) and 84 ng EEQ/L in wine (Klinge *et al.*, 2003). Moreover, levels of a few ng EEQ/L were measured in soy-based food (Takamura-Enya *et al.*, 2003).

In human breast milk, estrogen levels vary according to the stage of lactation from 34 to 124 ng/L (Adugamov and Chernikov, 1985).

Thomson & Grounds (2005) estimated that BPA would represent 7% of the estrogenic potential contributed by food, isoflavones accounting for 51%, alkylphenols 25% and flavonoids 17%.

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[11] Among 13 thermal printer papers analysed, 11 contained between 8 and 17 g/kg BPA (Biedermann S., Tschudin P., Grob K., Transfer of bisphenol A from thermal printer paper to the skin, *Analytical and Bioanalytical Chemistry, submitted manuscript).*

[12] Gene mutation assay on bacteria (negative), Chromosomal aberration in cultured mammalian cells (positive), Gene mutation assay on cultured mammalian cells (negative), Micronucleous assay (negative).

5. OTHER STUDIES ANALYSED BY THE BPA WG

The data reviewed by the BPA WG also included studies measuring concentrations in humans as well as articles or summary reports on risk assessment relating to BPA.

5.1 Data on concentrations in humans

5.1.1 GerES IV: Phthalate metabolites and bisphenol A in urine of German children

Becker K., Göen T., Seiwert M., Conrad A., Pick-Fuß H., Müller J., Wittassek M., Schulz C., Kolossa-Gehring M., *International Journal of Hygiene and Environmental Health* (2009) 212: 685-692.

Rating 2c

Objective:
To measure concentrations of BPA in 600 urine samples from German children.
Method:
From a cohort of 1790 German children aged 3 to 14 years, a subsample of 600 children was selected according to standard stratification variables (age group, gender, origin). The age groups used were: 3-5, 6-8, 9-11 and 11-13 years.
Considering that BPA is absorbed almost completely and virtually completely eliminated in urine in the form of glucuronic acid conjugate, the authors estimated that urine is the most suitable biological fluid for exposure assessment.
Total BPA was measured in morning urine after enzymatic hydrolysis of conjugates and derivisation by GC/MS-MS with internal standard and a limit of quantification of 0.15 µg/L.
Results:
BPA was detected in 99% of the samples. The median concentrations in the four age groups ranged between 2.1 and 3.5 µg/L, those of the 95th percentile between 11 and 23 µg/L. The maximum value was 205 µg/L and the average 2.74 µg/L. These results are consistent with the work of Calafat *et al.* (2008[13]) for the NHANES 2003-2004 study that indicated for children aged 6 to 11 years values of 3.6 µg/L on average and 16 µg/L at the 95th percentile. No gender difference was observed, however higher concentrations were measured in children aged 3-5 years compared to the cohort as a whole (geometric means: 3.55 and 2.66 µg/L, respectively).
Based on these concentrations and an estimated amount of urine, the median daily exposure would be 0.05 µg/kg b.w., or approximately 1000 times less than the TDI set by EFSA. At the 95th percentile, the expoure would be 0.37 or 0.22 µg/kg b.w. (depending on calculation assumptions) and the maximum 7 µg/kg b.w., also below the TDI.
Comments of the BPA WG:
A single morning urine sample from each individual was analysed. Given the rate of metabolism in humans (half-life of 4 to 5h according to Volkel et al., 2002) and the fact that intra-individual variation may also be as great as inter-individual variation, the results of this study provide qualitative (presence of BPA in humans) rather than quantitative information.

***To conclude, the BPA WG** considers that the results of this study provide evidence concerning BPA exposure in German children.*

5.1.2 Exposure to bisphenol A and other phenols in neonatal intensive care unit premature infants

Calafat A.M., Weuve J., Ye X., Jia L.T., Hu H., Ringer S., Huttner K., Hauser R., *Environmental Health Perspectives* (2009) 117: 639-644.

Rating 1d

Objective:
To measure concentrations of BPA in the urine of 41 premature infants from two hospitals in the United States (Boston).
Method:
Measurements were performed on the urine of 41 premature infants with a corrected gestational age (gestational age at birth plus age after birth) of ≤ 44 weeks. The urine samples were collected from the

[13] Calafat A.M., Ye X.., Wong L.Y., Reidy J.A., Needham L.L. (2008). Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. *Environ. Health Perspect.* 116(1):39-44.

cotton filling in nappies. Concentrations of free BPA and total BPA were measured using solid-phase extraction coupled with HPLC-MS. The detection limit was 0.4 µg/L.
Results:
The results show that during this critical phase of development, the babies were exposed to levels of BPA (free and conjugated) about one order of magnitude higher than that of the general population of children and adults (30 µg/L on average and almost 1000 µg/L maximum). A significant difference in BPA concentrations was observed in the urine from one hospital to another. Plastic medical devices were not analysed, but the correlation with Di(2-ethylhexyl) phthalate (DEHP) metabolites indicate that articles plasticised with PVC were the primary source of exposure.
Comments of the BPA WG:
The BPA WG calls attention to the fact that ***these data are specific to premature infants in intensive medical care and infused with medical devices containing BPA. They cannot be extrapolated to newborns in general.***
This study also shows that premature infants can metabolise BPA since 90% of the BPA was found in conjugated form in more than three-quarters of the premature infants.

To conclude, the BPA WG *considers that the results of this study demonstrate that the plastics used in medical devices can be a source of exposure to BPA and that BPA is metabolised in premature infants.*

5.1.3 Polycarbonate bottle use and urinary bisphenol A concentrations

Carwile J.L., Luu H.T., Bassett L.S., Driscoll D.A., Yuan C., Chang J.Y., Ye X., Calafat A.M., Michels K.B., *Environmental Health Perspectives* (2009) 117: 1368-1372.

Rating 3b

Objective:
To study the effect of the consumption of cold beverages contained in polycarbonate (PC) bottles on urinary BPA concentrations.
Method:
Seventy-seven students at Harvard College underwent a seven-day 'washout' phase during which their cold beverages were to be limited to those contained in stainless steel bottles. Control urine samples were collected on the second and third days of this period, between 1600 hours and 2000 hours. Polycarbonate (PC) bottles were then distributed to participants for one 'intervention' week and were to be used for all cold beverages. Urine was donated at two collection times between 1700 hours and 2000 hours over the last three days of this 'intervention' period.
Concentrations of free and conjugated BPA (after β-glucuronidase/sulfatase action) were measured by HPLC/MS-MS with a detection limit of 0.4 µg/L (100 µL urine test sample).
Results:
The BPA concentration was below the detection limit in nine samples from the washout week and three samples from the intervention week.
The geometric mean concentration of BPA was 1.2 µg/g creatinine (95% CI: 1.0 – 1.4) during the washout phase and 2.0 µg/g creatinine (95% CI: 1.7 – 2.4) during the intervention phase. The average increase in BPA concentration associated with polycarbonate bottle use was 69% (40 to 102%).
Study conclusion:
Beverages contained in PC bottles contribute to BPA exposure.
Comments of the BPA WG:
The nature of the beverages that were put in the bottles, the duration of contact time with the PC before consumption as well as the temperature of the drink were not specified. In fact, high temperature, pH and/or hardness are known factors that favour the release of BPA from polycarbonate (see previous section on exposure to BPA), particularly concerning beverages other than water.
The significance of this study is limited, especially since the authors did not exclude methodological bias such as stricter adherence to protocol during the washout phase in comparison with the intervention phase.

To conclude, the BPA WG *does not accept the results of this study as demonstrating the contribution of polycarbonate bottles to total intake of BPA, due to its methodological limitations. But the BPA WG considers that they provide evidence concerning BPA exposure in the American student population.*

5.1.4 Sensitive and selective method for the determination of bisphenol A and triclosan in serum and urine as pentafluorobenzoate-derivatives using GC-ECNI/MS

Geens T., Neels H, Covaci A., *Journal of Chromatography B* (2009) 877 (31): 4042-4046.

Rating 3a

Objective:
To develop and validate an analytical method for measuring concentrations of BPA and triclosan[14] in human urine and serum samples by GC-MS/MS.
Method:
Urine and serum BPA concentrations were measured in 20 Belgian adolescents. The urine or serum samples were subjected to digestion by β-glucuronidase / sulfatase in order to measure total BPA (free and conjugated). After solid-phase extraction, the samples were derivatised prior to being analysed by GC-MS/MS. The quantification limits were 0.5 and 0.2 µg/L in the serum and urine, respectively.
Results:
BPA was detected in 100% of urine samples. The median values found for urinary BPA were 1.25 µg/L (values ranged from 0.58 to 5.2 µg/L). The serum BPA concentrations (only 10% of samples were positive) ranged between < 0.5 µg/L and 0.59 µg/L.
Study conclusion
The method that was developed and validated enabled the simultaneous determination of BPA and triclosan, and urine appeared to be a more appropriate matrix for biomonitoring studies.
Comments of the BPA WG:
The main topic of the article is methodological. The BPA concentrations measured cannot be interpreted because of the limited number of samples analysed and incomplete demographic data (origin, sex and age).

***To conclude, the BPA WG** considers that the results of this study provide evidence concerning BPA exposure in the Belgian adolescent population.*

5.1.5 Levels of metabolites of organophosphate pesticides, phthalates, and bisphenol A in pooled urine specimens from pregnant women participating in the Norwegian Mother and Child Cohort Study (MoBa)

Ye X., Pierik F.H., Angerer J., Meltzer H.M., Jaddoe V.W.V., Tiemeier H., Hoppin J.A., Longnecker M.P., *International Journal of Hygiene and Environmental Health* (2009) 212: 481–491.

Rating 3b

Objective:
To measure several contaminants including BPA in the urine of pregnant women in Norway. The results are enhanced by two similar studies: the Generation R study (Jaddoe *et al*., 2006[15]) conducted in the Dutch city of Rotterdam in which the urine of 100 pregnant women was collected and measured for BPA, and the NHANES survey (CDC, 2008[16]) in the United States in which BPA concentrations were measured in the urine of 87 pregnant women.
Method:
As part of a national programme started in 1999, blood and urine was collected from pregnant women during a routine ultrasound examination performed at the 17th or 18th week of pregnancy. Urine from 110 women was pooled, with 11 urine samples per pool. The amount of BPA absorbed per day was determined by the following formula: (Concentration in urine x Volume of urine) / body weight. The urine volume was estimated at 2 litres (Branstaeter *et al*., 2009[17]) and the average body weight was estimated at 73.5 kg.

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[14] Triclosan: an anti-microbial used in cosmetic products and suspected of inducing microbial resistance.

[15] Jaddoe V.W., Mackenbach J.P., Moll H.A., Steegers E.A., Tiemeier H., Verhulst F.C., Witteman J.C., Hofman A. (2006). The generation R study: design and cohort profile. *Eur. J. Epidemiol.* 21(6): 475-484.

[16] CDC (2008). National Health and Nutrition Examination Survey Data. US Department of Health and Human Services, Centers for Disease Control and Prevention, Hyattsville, MD. Available from http://www.cdc.gov/nchs/about/major/nhanes/datalink.htm, accessed on 22-Jan-2010.

[17] Brantsaeter A.L., Haugen M., Julshamn K., Alexander J., Meltzer H.M. (2009). Evaluation of urinary iodine excretion as a biomarker for intake of milk and dairy products in pregnant women in the Norwegian mother and child cohort study (MoBa). *Eur. J. Clin. Nutr.* 63:347-54.

Results:
The mean concentration of BPA in the pooled urine of 110 pregnant women was 4.5 µg/L which is near the levels measured in the two other studies referenced: 2.52 µg/L in the Generation R study (a significant difference compared with the Norwegian results) and 3.93 µg/L in the NHANES study (no significant difference).
Study conclusion:
The maximum daily intakes that the authors derived from the data collected in Norway were 0.1 µg/kg b.w./d, well below the TDI set by EFSA (0.05 mg/kg b.w./d).
Comments of the BPA WG:
The analytical method used for BPA was not described. The pooling of the urine has introduced several biases, mainly that of being unable to account for the measure of individual concentrations. The calculation of daily intake was also biased, especially since default values were used for the daily urine volume and weight.

***To conclude, the BPA WG** nevertheless accepts that the results of this study provide evidence concerning BPA exposure of the Norwegian pregnant women population.*

5.2 Articles or review reports on BPA risk assessment

The BPA WG has examined the articles and review reports on BPA risk assessment listed below. Given the nature of these data, they are not summarised in this annex.
These data were used to prepare an overview of various international positions on health risk assessment linked to BPA and to identify critical points within the scientific community (metabolism in humans and particularly in newborns, adequacy of standardised toxicology tests to characterise the toxicity of endocrine disruptors, the hypothesis of a non-monotone dose-response relationshipwith higher toxicity at low doses).

Risk to all or none? A comparative analysis in the health risk assessment of bisphenol A
Beronius A., Ruden C., Hakansson H., Hanberg A., *Reproductive Toxicology* (2010) 29: 132–146.

Health risk assessment procedures of endocrine disrupting compounds within different regulatory frameworks in the European Union
Beronius A., Ruden C., Hanberg A., Hakansson H., *Regulatory Toxicology and Pharmacology* (2009) 55: 111-122.

A CASCADE of effects of bisphenol A
Bondesson M., Jönsson J., Pongratz I., Olea N., Cravedi J.P., Zalko D., Håkansson H., Halldin K., Di Lorenzo D., Behl C., Manthey D., Balaguer P., Demeneix B., Fini J.B., Laudet V., Gustafsson J.A., *Reproductive Toxicology* (2009) 28: 563-567.

Does rapid metabolism ensure negligible risk from bisphenol A?
Ginsberg G. and Rice D.C., *Environmental Health Perspectives* (2009) 117: 1639-1643.

Bisphenol A workshop of German Federal Environment Agency – March 30-31, 2009. Work group report: public health issues of bisphenol A.
Gies A., Heinzow B., Dieter H.H., Heindel J., *International Journal of Hygiene and Environmental Health* (2009) 212: 693-696.

BPA: traditional toxicology testing is inadequate and concerns extend beyond aneuploidy.
Hunt P.A. and Hassold T., *Trends in Genetics* (2009) 25: 15-16.

Why public health agencies cannot depend on good laboratory practices as a criterion for selecting data: the case of bisphenol A.
Myers J.P., vom Saal F.S., Akingbemi B.T., Arizono K., Belcher S., Colborn T., Chahoud I., Crain D.A., Farabollini F., *et al. Environmental Health Perspectives* (2009) 114: 309-315.

Evidence on the developmental and reproductive toxicity of bisphenol A
OEHHA (Office of Environmental Health Hazard Assessment, California Environmental Protection Agency, Reproductive and Cancer Hazard Assessment Branch), Draft May 2009 (297 p), final version October 2009 (302 p) and analysis of comments from the public consultation.

Chapel Hill bisphenol A expert panel consensus statement: Integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure
Vom Saal F.S., *Reproductive Toxicology* (2007) 24: 131–138.

References from the published scientific literature concerning bisphenol A, focusing on "low dose" *in vivo* effects, molecular mechanisms based primarily on *in vitro* studies, sources of exposure and pharmacokinetics
Vom Saal F.S., August 2008, 164p, http://endocrinedisruptors.missouri.edu/vomsaal/vomsaal.html

Abbreviations

AFSSA: French Food Safety Agency [Agence Française de Sécurité Sanitaire des Aliments]
BPA: Bisphenol A
BPA WG: AFSSA 'Bisphenol A' working group
b.w.: body weight
CEDI: Cumulative Estimated Daily Intake
CNS: Central Nervous System
DES: Diethylstilbestrol
ED: Endocrine Disruptor
EE_2: Ethinyl estradiol
EFSA: European Food Safety Authority
EMDI: Estimated Maximum Daily Intake
ER: Estrogen Receptor
F1: 1^{st} generation
F2: 2^{nd} generation
F3: 3^{rd} generation
FCMs: Food Contact Materials
GC: gas chromatography
GD: Gestational day
GLP: Good Laboratory Practices
GnRH: Gonadotropin Releasing Hormone
HPLC: High performance liquid chromatography
LOAEL: Lowest observed adverse effect level
MOE: Margin of exposure
MS: Mass spectrometry
NOAEL: No observed adverse effect level
PC: Polycarbonate
PND: Postnatal Day
TDI: Tolerable Daily Intake
US EPA: United States Environmental Protection Agency
US FDA: United States Food and Drug Administration

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