

IMPACT OF BUTYL BENZYL PHTHALATE ON DEVELOPMENT OF THE REPRODUCTIVE SYSTEM OF EUROPEAN PIKEPERCH, *SANDER LUCIOPERCA* (L.)

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The effect of butyl benzyl phthalate (BBP) on the sex differentiation process of fish is practically unknown. The experimental material of this study was juvenile European pikeperch [*Sander lucioperca* (L.)], which is gonochoristic, undergoes immediate sex differentiation, and has a fixed gonad differentiation period. The fish were fed a diet supplemented with BBP (during the sex differentiation phase: age 61–96 days post hatch) in the following quantities: 1.0; 2.0; 4.0; 8.0; 16.0 g BBP kg⁻¹ feed. The control feed was a xenobiotic-free base feed. In the present experiment lasting 10 weeks, the survival and growth of fish, the histopathological changes of the fish gonads and the sex ratio were evaluated. After administration of the two highest doses of BBP, growth inhibition of the fish was observed. BBP also seriously disturbed the gonadal differentiation process of pikeperch. All analysed concentrations of BBP delayed testicular development and, at concentrations of 4.0, 8.0 and 16.0 g BBP kg⁻¹, induction of the feminisation process was observed. The sex ratio was distinctly disrupted in groups receiving 8.0 and 16.0 g BBP kg⁻¹.

Key words: Xenobiotics, endocrine disruptive chemicals, phthalates, butyl benzyl phthalate, gonadal development, pikeperch

Phthalates, which are derivatives of phthalic acid, salts or esters, are used in the production of paints, lacquers, solvents, and as plasticisers in the manufacture of polyvinyl chloride (PVC) products. Phthalates enter the environment and the human and animal food webs because of their wide-ranging application in industry and their physicochemical properties. In the 1990s, human reproductive problems began to be associated with the occurrence of phthalates in the environment (Swain and Walton, 1990). Studies on rodents demonstrated that mainly dialkyl phthalates containing from 4 to 6 carbon atoms in the alkyl chain, including butyl benzyl phthalate (BBP) can potentially affect endocrine function (e.g. Tyl et al.,

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2004). The oestrogenic activity of the esters (i.e. competitive displacement of 17β -oestradiol and binding with oestrogen receptors) has been documented in many *in vitro* studies (e.g. Harris et al., 1997). During *in vivo* assays, after receiving 500 mg kg^{-1} IP injections of BBP, rainbow trout (*Oncorhynchus mykiss*) were observed to synthesise vitellogenin, a protein indicator of oestrogenic endocrine disruptive chemicals (EDCs) (Christiansen et al., 1998). On the other hand, by *in vitro* studies of yeast cell cultures Sohoni and Sumpter (1998) demonstrated that BBP exhibits strong anti-androgenic activity by blocking androgen receptors (AR). Low but significant elevation in the expression of AR was observed in the developing embryos of fathead minnows (*Pimephales promelis*) after BBP exposure (Mankidy et al., 2013). There are increasing numbers of reports that xenobiotics, which were initially classified as oestrogenic compounds, are either exclusively or additionally AR antagonists. Most studies of the biological effects of phthalates (including BBP) that are conducted *in vivo* on mammals indicate that there is disruption in the development of the male reproductive system, including in the epididymis, vas deferens, seminal vesicles, prostate, external genitalia, and the demasculinisation of the male fetus manifested in shortened anogenital distance (Gray et al., 2000). Recent studies with zebrafish (*Danio rerio*) demonstrated that BBP at environmentally relevant concentrations induces changes in sperm motility in adult males (Oehlmann et al., 2009). These malformations are associated with lower testosterone (T) levels and provide a strong support for an anti-androgen mode of action of phthalate esters (David, 2006).

To date, apart from acute toxicity tests, the impact of phthalates on fish and their reproduction practically has not been studied. The low water solubility of BBP, about 2.7 mg dm^{-3} , and the quick metabolism of low dosages of phthalates (Staples et al., 1997) do not permit evaluating whether or not or to what degree phthalates disrupt fish reproduction. What appears to be more justifiable in this case is to conduct studies *per os*. Mackintosh et al. (2004) analysed 14 species of aquatic organisms from False Harbor, Vancouver, British Columbia, which contained from 1.61 to $2.90 \text{ } \mu\text{g BBP kg}^{-1}$ bw. Seaperch (*Embiotoca laterali*) from the waters of False Creek, Vancouver, Canada contained up to $10 \text{ } \mu\text{g BBP kg}^{-1}$ bw (Lin et al., 2003). Györkös (1996) reports concentrations of BBP in fish from British rivers that range from 0.05 to $39.0 \text{ } \mu\text{g kg}^{-1}$ bw. Further, the tissues of sea lamprey (*Petromyzon marinus*) from Brodhead Creek in Pennsylvania had concentrations of approximately $710 \text{ } \mu\text{g BBP kg}^{-1}$ (Stephanatos and Knorr, 1992). The analyses of fish available for retail sale on the Hong Kong market indicate high levels of phthalate esters. The phthalate concentrations in freshwater fish from this region ranged from 1.66 to 3.14 mg kg^{-1} bw, while those in marine fish were higher, ranging from 1.57 to 7.10 mg kg^{-1} (Cheng et al., 2013).

The aim of the current study was to identify the effect of BBP at concentrations of 1.0 , 2.0 , 4.0 , 8.0 and 16.0 g kg^{-1} feed on the development of the reproductive systems and the growth of European pikeperch [*Sander lucioperca* (L.)].

Pikeperch is gonochoristic, undergoes immediate sex differentiation, and has a fixed gonad differentiation period (Demska-Zakęś and Zakęś, 1995).

Materials and methods

Fish and experimental design

The juvenile European pikeperch used in the experiment were obtained through out-of-season artificial reproduction (according to procedures developed previously; Zakęś, 2007) performed in the winter, in January. The larvae were reared initially at the Department of Sturgeon Breeding at Pieczarki, Inland Fisheries Institute (IFI) in Olsztyn. When the fish had reached a body weight of about 1.5 g, they were transported to the Department of Aquaculture at IFI in Olsztyn. After a two-week adaptation period the fish were sorted. The experiment was performed on 240 individuals with body weights ranging from 1.00 to 2.20 g (61 days post hatch, DPH, Table 1), and placed in six circulating tanks with a working volume of 28 dm³ (stocking density: 2.2 ± 0.05 g dm⁻³). The experiment was divided into two stages and ran for ten weeks. During this time the physicochemical parameters of the water were monitored. Water temperature was 21.7 ± 0.2 °C, oxygen content at the outlet was 7.66 ± 0.11 mg O₂ dm⁻³, the contents of ammonia nitrogen and nitrite-nitrogen at the outlet were 0.035 ± 0.012 mg TAN dm⁻³ and 0.047 ± 0.032 mg NO₂-N dm⁻³, respectively. The water pH ranged from 7.5 to 7.7. Light intensity at the water surface was 50–60 lx. Water flow through the individual tanks was 1.5 ± 0.3 dm³ min⁻¹.

Feed and feeding

During the first stage of the experiment that ran for five weeks (61–96 DPH), the fish were fed a base feed (NUTRA, Trouvit, Nutreco Aquaculture, France) with the addition of butyl benzyl phthalate (Sigma-Aldrich, Poland) at concentrations of 1.0, 2.0, 4.0, 8.0 and 16.0 g BBP kg⁻¹ feed. A measured volume of the master solution of phthalate and 96% ethyl alcohol was diluted in 6 cm³ of 96% ethyl alcohol and mixed into 100 g of feed. The supplement was added to the feed using pressure with a vacuum device by AGA Labor (Lublin, Poland), and then dried for 24 h at room temperature. The control was the base feed with solvent. The fish were fed a commercial feed exclusively during the second stage of the experiment that also ran for five weeks (97–132 DPH). During the experiment, the feed was delivered continuously (for 18 h daily) with a 4305 FIAP automatic band feeder (Fishtechnik GmbH, Germany). As the fish grew, the daily feed dose was reduced successively from 5 to 2% of the stock biomass. Fish mortality was monitored daily.

Table 1
Rearing indices at the beginning and after stage I (96 DPH) and II (132 DPH) of the experiment (mean \pm SD)

| | Butyl benzyl phthalate concentrations (g kg ⁻¹ feed) | | | | | |
|----------------------------------|---|------------------|------------------|------------------|-------------------|-------------------|
| | 0 | 1.0 | 2.0 | 4.0 | 8.0 | 16.0 |
| Initial body weight (g) | 1.80 \pm 0.26 | 1.80 \pm 0.28 | 1.79 \pm 0.22 | 1.77 \pm 0.25 | 1.82 \pm 0.25 | 1.76 \pm 0.28 |
| Body weight after 96 DPH (g) | 8.41 \pm 1.89 | 7.84 \pm 1.50 | 7.30 \pm 1.36 | 6.76 \pm 1.41* | 6.57 \pm 1.13* | 4.31 \pm 0.77* |
| Body weight after 132 DPH (g) | 18.14 \pm 4.89 | 17.42 \pm 4.68 | 15.46 \pm 4.63 | 14.84 \pm 4.33 | 13.48 \pm 3.19* | 10.23 \pm 3.00* |
| Initial total length (cm) | 5.89 \pm 0.34 | 5.93 \pm 0.34 | 6.01 \pm 0.29 | 5.97 \pm 0.34 | 6.15 \pm 0.29 | 5.97 \pm 0.31 |
| Total length after 96 DPH (cm) | 9.97 \pm 0.79 | 9.81 \pm 0.70 | 9.62 \pm 0.55 | 9.35 \pm 0.69* | 9.43 \pm 0.50* | 8.25 \pm 0.49* |
| Total length after 132 DPH (cm) | 13.10 \pm 1.03 | 12.94 \pm 1.09 | 12.24 \pm 1.02 | 12.70 \pm 1.86 | 12.13 \pm 0.96 | 11.10 \pm 0.86* |
| Initial condition factor K | 0.88 \pm 0.16 | 0.86 \pm 0.07 | 0.83 \pm 0.07 | 0.83 \pm 0.09 | 0.78 \pm 0.07 | 0.82 \pm 0.08 |
| Condition factor K after 96 DPH | 0.84 \pm 0.05 | 0.82 \pm 0.06 | 0.81 \pm 0.06 | 0.82 \pm 0.07 | 0.78 \pm 0.06* | 0.76 \pm 0.05* |
| Condition factor K after 132 DPH | 0.79 \pm 0.05 | 0.78 \pm 0.08 | 0.78 \pm 0.15 | 0.81 \pm 0.05 | 0.76 \pm 0.03 | 0.73 \pm 0.03 |

*statistically significant differences in relation to the control group (P < 0.05)

Experimental procedures

After the conclusion of stages I and II of the experiment the fish were weighed (± 0.01 g) and measured (± 0.1 cm). The fish were anaesthetised prior to all manipulations in a solution of etomidate (Propiscin, IFI, Olsztyn). The data obtained were used to calculate the fish condition factor $K = (bw \cdot 100) \cdot Lt^{-3}$; where: bw – body weight (g), Lt – total length (cm).

At the beginning of the experiment (61 DPH) and after stages I and II, the gonads of 15 individuals from each group were collected for histological analyses (procedures described by Demska-Zakęś, 2005). The degree of gonad development, histological changes, and sex ratio were also evaluated.

The mean values of the rearing indexes were compared with Leven's test, ANOVA single factor variance of analysis, and Tukey's post hoc test. The sex ratio was verified with the test of two structure indicators, while the dependence between phthalate concentration and the number of males was verified with Spearman's rank correlation coefficient. All of the tests were verified at a level of significance of $P < 0.05$ using the STATISTICA 8.0 program.

Results

Butyl benzyl phthalate did not have a significant impact on fish survival. After stage I of the experiment, it was noted that increasing doses of the xenobiotic in the feed were linked to decreases in pikeperch weight gain and body length growth. As a result, the fish fed the feed with the highest dose of BBP weighed nearly half as much as the fish from the control group (4.31 in comparison to 8.41 g, respectively). The suppressive impact of this toxin on weight gain rates was also noted in the fish exposed to 4.0 and 8.0 g BBP kg^{-1} (Table 1). The total length of the fish was also the lowest in the group fed 16.0 g BBP kg^{-1} . Although the fish received no phthalates in the second stage of the study, similar tendencies were noted. Pikeperch from groups 8.0 and 16.0 g BBP kg^{-1} grew the least. The condition of fish 96 DPH fed 8.0 and 16.0 g BBP kg^{-1} was significantly lower ($P < 0.05$). After stage II of the experiment, no inter-group differences were noted in fish condition (Table 1).

The 96 DPH females from the control group had paired, cystic ovaries filled with oogonia and oocytes in prophase I of meiotic division. Well-developed oviducts were also observed. The male reproductive system comprised fusiform testes that were much smaller than the ovaries; the male gonads comprised gonocytes, numerous seminal vesicles and spermatogonia, and a partially developed sperm duct. Immediately after the conclusion of intoxication with doses of 1.0 and 2.0 g BBP kg^{-1} , the sex ratio of females to males was similar to that in the control group at 46.67 and 53.3%, respectively (Fig. 1). However, in these groups in 50 and 75% of the males, respectively, there were distinctly smaller gonads

with a reduced number of seminal vesicles and spermatogonia. The female reproductive system developed normally and similarly to that in the control group. With increasing concentrations of BBP in the feed, the percentage of males decreased ($r_s = -0.8804$; $P < 0.05$). In the groups given a feed supplemented with 4.0, 8.0 and 16.0 g BBP kg^{-1} , there were 1, 2, and 1 bisexual individuals, respectively. The histological picture of these gonads showed differentiated testes containing spermatogonia and a few oogonial cells. The sex ratio was distinctly disrupted in groups receiving 8.0 g BBP kg^{-1} ($P < 0.01$) and 16.0 g BBP kg^{-1} ($P < 0.05$) (Fig. 1). In the groups 4.0, 8.0 and 16.0 g BBP kg^{-1} , delayed testis differentiation was observed in 66.67 to 75% of the males, and 1, 3 and 3 individuals, respectively (from 12.5 to 30%), identified as females, had atypical ovaries that were significantly smaller and had an increased amount of connective tissue. The oviducts failed to develop.

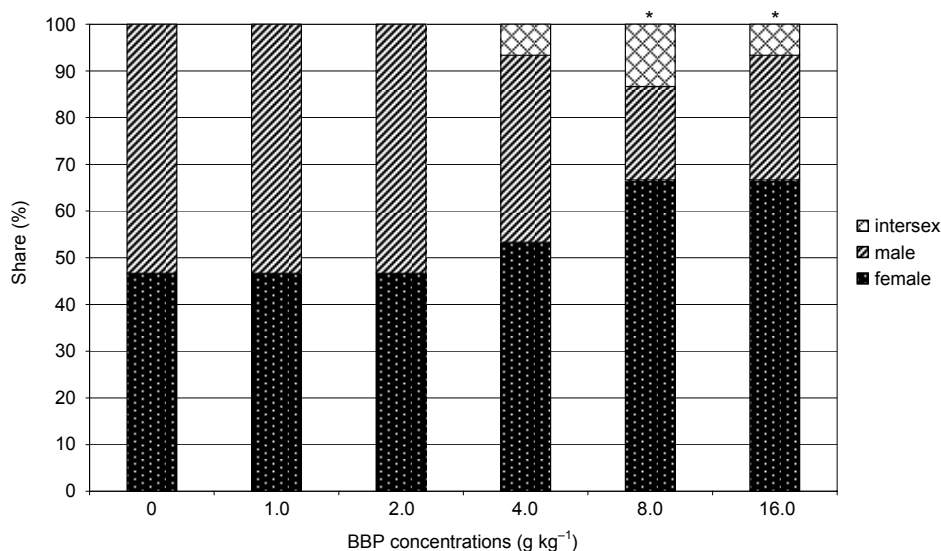


Fig. 1. Sex ratio of pikeperch in the control group and in groups exposed to butyl benzyl phthalate (BBP) after the first stage of the experiment (96 days post hatch, DPH). The asterisk (*) denotes significant statistical differences in sex ratio ($n = 15$; $P < 0.05$)

After the subsequent five weeks of rearing, the 132 DPH females comprising about 50% of the population had distinctly developed oviducts and relatively large ovaries. Within the differentiated ovarian basal lamella there were numerous previtellogenic oocytes, while there were sporadic oocytes in prophase I of meiotic division and oogonia. The males presented with weakly differentiated sperm duct and the pear-shaped testes were filled with numerous seminal vesicles with spermatogonia and first order spermatocytes.

Anomalies in the reproductive system of fish and changes in the sex ratio were similar to those following stage I of the experiment. All of the BBP-treated groups from 55.6% (group 1.0 g BBP kg⁻¹) to finally 100% of the males (group 16.0 g BBP kg⁻¹) had substantially smaller testes in comparison with the 132 DPH pikeperch from the control group. Additionally, in groups 4.0, 8.0 and 16.0 g BBP kg⁻¹, 6.7, 13.3 and 6.7% of individuals, respectively, presented with bisexual gonads. Single female sex cells were visible in the histological picture of testicular tissue. In the same groups (4.0, 8.0 and 16.0 g BBP kg⁻¹) 70–88.9% of the females had properly developed gonads, while the remaining individuals had smaller ovaries with undeveloped oviducts. As the concentration of BBP in the feed increased, the number of males in the collected samples decreased ($r_s = -0.9276$; $P < 0.05$). Significant sex ratio imbalance was observed in fish that received the two highest doses of BBP in stage I of the experiment, i.e. in groups 8.0 ($P < 0.01$) and 16.0 g BBP kg⁻¹ ($P < 0.05$) (Fig. 2).

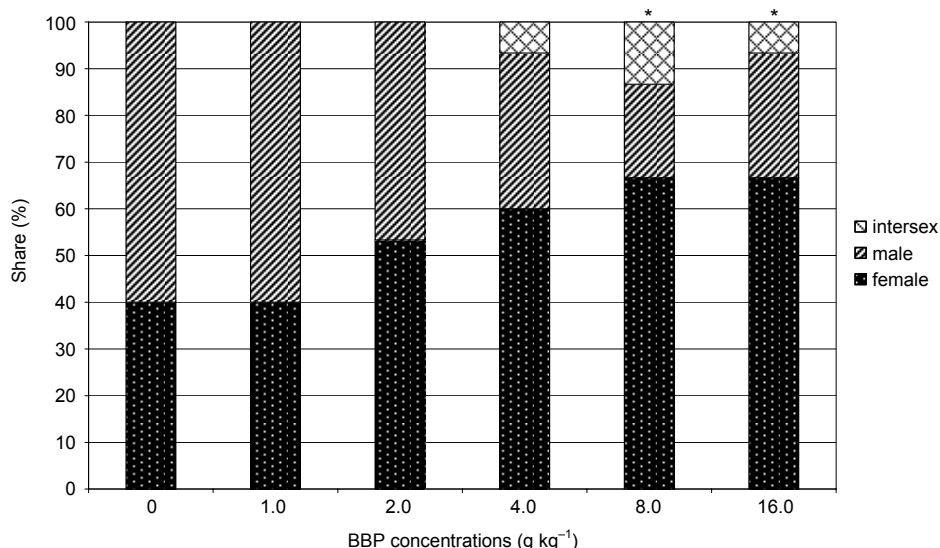


Fig. 2. Sex ratio of pikeperch in the control group and in groups exposed to BBP after the second stage of the experiment (132 DPH). The asterisk (*) denotes significant statistical differences in sex ratio ($n = 15$; $P < 0.05$)

Discussion

After the pikeperch received the two highest doses of butyl benzyl phthalate in their feed, i.e. 8.0 and 16.0 g BBP kg⁻¹, their growth was halted permanently. This might indicate damage to the organs that participate in the detoxification of this compound. After mammals are exposed to phthalates, a range of

pathological responses are observed in the liver, pancreas, kidneys and spleen (reviewed by Kavlock et al., 2002), which, in the case of fish, might signal not only a disruption in metabolic but also in haematopoietic and immune processes.

Exposing pikeperch to BBP during the period when their bodies are most sensitive to steroids, i.e. during sexual differentiation, seriously disrupted the gonad development process. All of the BBP concentrations analysed retarded the development of pikeperch testes. At the conclusion of the experiment, this was noted in 50% of the males that had received the lowest concentration of BBP, and as the phthalate concentration in the feed increased, the percentage of males with poorly developed testes also increased (up to 100% of the males in the group that received 16.0 g BBP kg⁻¹). A few instances of intersex individuals were noted at concentrations of 4.0, 8.0 and 16.0 g BBP kg⁻¹. Since pikeperch is a gonochoristic species with stable sexual function, it can be concluded that BBP had caused sex inversion in a few of the males that had been exposed to these concentrations of BBP as was evidenced by the small ovaries similar in size to the testes but which lacked oviducts. This type of gonad was not noted in the control group. Moreover, BBP seriously affected the sex ratio at concentrations of 8.0 and 16.0 g BBP kg⁻¹. Sex inversion and hermaphroditism do not occur in gonochoristic species under natural environmental conditions, which is why the changes observed in pikeperch should be interpreted as the result of the endocrine disrupting activity of phthalates.

Similar changes, such as the induced development of an ovotestes or sex inversion, were observed after the pikeperch had been exposed to exogenous xeno-oestrogen nonylphenol and 17β-oestradiol (Demska-Zakęś, 2005). Interestingly, symptoms of the feminisation of fish gonads and the inhibition of spermatogenesis were observed in medaka (*Oryzias latipes*) after exposure to the fungicide vinclozolin, which is known to function as an androgen receptor antagonist (Kiparissis et al., 2003).

Another ester, di-n-butyl phthalate (DBP), induced the occurrence of intersex specimens and sex inversion in studies conducted on genetic males of the Japanese wrinkled frog (*Rana rugosa*) (Ohtani et al., 2000). Also, following exposure to DBP, the African clawed frog (*Xenopus laevis*), exhibited delayed spermatogenesis and symptoms of feminisation manifesting as well-developed fallopian tubes in the testes (Lee and Veeramachaneni, 2005). Three percent intersex individuals were observed among Atlantic salmon (*Salmo salar*) exposed to the effects of di-2-ethylhexyl phthalate (DEHP) at concentrations of 1.5 g kg⁻¹ feed (Norman et al., 2007). In other studies, also conducted on salmon, the same dose of 1.5 g DEHP kg⁻¹ caused statistically significant increases in the percentage of females (64%; Norrgren et al., 1999). A dose of 5.0 g DEHP kg⁻¹ in the feed substantially disrupted spermatogenesis in adult male zebrafish (*Danio rerio*) (Uren-Webster et al., 2010).

There are growing numbers of reports indicating that anti-androgens might play a crucial role in the artificially induced feminisation of male reproductive systems (Kelce and Wilson, 1997). During competitive binding with AR and the blocking of endogenous androgen action, anti-androgens can create 'an oestrogenic environment', which exhibits symptoms of oestrogen exposure (Sohoni and Sumpter, 1998). Thus, oestrogen and anti-androgens can produce similar phenotypic responses in organisms even if they act through different mechanisms. The molecular foundation for the feminisation effect of androgens and oestrogens in fish is not well understood. Filby et al. (2007) studied the expression of 22 genes (linked to processes controlled by androgens and oestrogens, i.e. reproduction, growth, maturation) localised in the livers and gonads of female and male fathead minnows. Studies conducted *in vivo* indicated that exposing this species to model androgen receptor antagonist flutamide and synthetic oestrogen 17 β -ethinyloestradiol (EE2), induced similar phenotype effects signalling feminisation. The results of *in vitro* studies indicated that both of these substances could induce the expression of these same genes, which demonstrates that similar mechanisms are at work.

The impact of phthalates on the reproduction of teleost fish has virtually not been investigated to date, and to the best of the authors' knowledge, the current study is the first to report that BBP disrupts sex differentiation processes in fish. Additionally, the authors have demonstrated that studies *per os* might also prove more applicable when studying the impact of phthalates on the reproductive systems of fish than immersion studies, since the former disallow the detoxification of these compounds on fish gills (Barron et al., 1989).

Fish are extremely sensitive model organisms in studies of reproductive toxicity. The pikeperch were given 0, 1.0, 2.0, 4.0, 8.0, 16.0 g BBP kg⁻¹, and the doses the fish ingested were 0, 45, 90, 180, 360, 720 mg kg⁻¹ bw d⁻¹ gross, respectively. The first change in the reproductive system of the fish, which was a delay in testicular development, was observed at the lowest dose of 45 mg kg⁻¹ bw d⁻¹ gross, while intersex individuals occurred at 180 mg kg⁻¹ bw d⁻¹ gross. Reproductive studies of rats indicated that the no-observed-effect level (NOEL) was 200 mg kg⁻¹ bw d⁻¹, while the first visible changes in the reproductive system of these rodents were not noted until a dose of 1000 mg kg⁻¹ bw d⁻¹ (reviewed by Kavlock et al., 2002).

The present study indicated that BBP can potentially disrupt endocrine system function in fish. It also confirmed that fish are more sensitive biomarkers of toxic substances than are mammals. BBP probably do not have a negative impact on fish reproduction at the levels at which they occur in the natural environment. In this context, however, it appears prudent to perform long-term, systematic studies of lower concentrations of phthalates. It is also necessary to track the impact other phthalates have on fish reproduction, to study the impact of phthalate mixtures since these are what mainly occur in the natural environment, and to perform multi-generational studies.

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