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1 Environmental pollution by bisphenol A: sources and fate in the Elbe basins and biological effects

Umweltverschmutzung durch Bisphenol A: Einträge und Stoffverhalten im Elbe-Einzugsgebiet sowie biologische Wirkungen

Key words: bisphenol A, emission, retention, endocrine disruptor, *Xenopus laevis*, thyroid system, reproductive system

Abstract

Households and industrial discharges are the main sources of bisphenol A (BPA), an environmental chemical suspected to cause severe effects on endocrine systems, in surface waters. The emissions are realised by waste water treatment plants (WWTP) and combined sewer systems as well as industrial direct discharges. It was estimated that the total inputs into the river system of Elbe are about 970 kg/a by WWTP, 70 kg/a by sewer systems and 510 by two industrial discharges in the Czech part of Elbe. The retention within the surface waters of Elbe is 790 kg/a or 51%. *Xenopus laevis* premetamorphic tadpoles at stages 48 and 51 were exposed to different BPA concentrations ranging from 223 ng/L to 223 µg/L to analyse effects on sexual differentiation and thyroid system. BPA caused moderate effects on thyroid system by interference with thyroid receptors but had adverse effects on sexual differentiation disrupting normal gonadal development particularly in males as shown by gross morphological and histological determinations.

Zusammenfassung

Haushalte und industrielle Direkteinleiter gehören zu den Haupteinträgern von Bisphenol A (BPA) in die Oberflächengewässer des Elbe-Einzugsgebietes. Die Emission erfolgt dabei über kommunale Kläranlagen (WWTP), Gemischtwasserkanalisation sowie industrielle Direkteinleiter. Der Gesamteintrag von BPA über die WWTP beläuft sich auf etwa 970 kg/a, der über die Gemischtwasserkanalisation auf 70 kg/a und etwa 510 kg/a entfallen auf zwei industrielle Direkteinleiter im tschechischen Teil der Elbe. Die Retention innerhalb der Oberflächengewässer der Elbe beträgt 790 kg/a bzw. 51%. *Xenopus laevis* Kaulquappen im Entwicklungsstadium 48 bzw. 51 wurden mit verschiedenen BPA-Konzentrationen im Bereich von 223 ng/L bis 223 µg/L exponiert, um die Wirkungen von BPA auf die Sexualdifferenzierung und das Schilddrüsensystem zu untersuchen. BPA beeinflußte das Schilddrüsensystem über Interaktionen mit dem Schilddrüsenrezeptor nur moderat, zeigte aber adverse Auswirkungen auf die Sexualdifferenzierung durch Störungen bei der Gonadenentwicklung von männlichen Individuen auf morphologischer und histologischer Ebene.

1.1 Introduction

Climate change is recently one of the most important issues in environmental sciences. Many scientific groups are concerned about worldwide temperature and precipitation changes within near future. However, not only rising temperatures and increase of natural disaster are able to modify regions, nutrients, animal or human populations but also environmental pollution in surface water. Since few years especially xenobiotica and recently pharmaceutical compounds are objects of ecotoxicological studies. Xenobiotica contain to the group of "endocrine disruptors" (ED). ED are compounds which interfere with endocrine systems and disrupt their normal functions within an organism without remarkable toxicity (Colborn et al. 1996, Kloas 2002). Since the 1990`s an increasing pollution of such compounds was noticed for instance in surface waters, agricultural areas, and atmosphere, especially since the analytical methods for detection have been continuously improved.

Bisphenol A (BPA) is one of the most common chemicals for production of epoxy resins and polycarbonate plastics (Fig. 1).

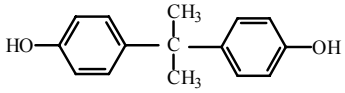

Fig. 2: Chemical structure of bisphenol A.

Abb. 2: Chemische Struktur von Bisphenol A.

BPA is widely used for all kind of products like computer housings, carpets, upholstery, for car paintings and flame retardants such as tetrabromobisphenol A (TBBA). BPA attracted public attention when it has been generally known that it can leach out of plastic baby bottles or cans and migrate into milk and food (Goodson et al. 2004, Braunrath et al. 2005). Since BPA could be detected in diverse human tissues and environmental samples it is nessessary to analyze its biological effects. Clear evidences exist for feminization caused by BPA in snails, fish, and amphibians (Oehlmann et al. 2005, Yokota et al. 2000, Levy et al. 2004) and many publications have shown effects regarding reproduction but some findings are still controversely discussed (Pickford et al. 2003, Yoshida et al. 2004).

To get a better understanding of BPA in a prospective perspective it is necessary to assess the general sources and environmental fate of BPA. Therefore the Elbe River system was used as a model. It is the second-largest river system in Germany and one of the largest in central Europe. The biological effects on thyroid system and reproduction were evaluated by means of an amphibian model, the South African clawed toad *Xenopus laevis*.

1.2 Material and Methods

Bisphenol A emissions and retention

The Elbe river system has been investigated for BPA emission and retention. Literature and internet were used to collect informations about chemical

properties, and usage of BPA as well as to accumulate data about BPA determination of surface water samples. Mean BPA emissions have been calculated by using the MONERIS model (Behrendt et al. 2003). This model considers following 7 different pathways: particle entry via erosion, dissolved entry via avulsion, basis flow, interflow, tile drainage, atmospheric deposition on water surface areas, sealed urban areas, and point source discharges.
Based on informations about inflow and outflow of several WWTP BPA retention for WWTP was calculated. The following retention function was used to create a graph which compares observed BPA concentrations measured along the Elbe main stream and calculated BPA concentrations:

$$\frac{C_{BPA}}{C_{BPA_{INPUT}}} = \frac{1}{\left(1 + a \cdot HL^{-1}\right)}$$

HL: Hydraulic loading
a: 7.18

BPA determination of water samples of Elbe-river catchment by HPLC

Additional to existing environmental data several water samples within the Elbe catchment and tributaries were collected to determine BPA in surface water. Water samples were taken monthly from Spree, Havel, Havel/Spree as well as from WWTP outflow Münchehofe and Erpe. Samples from Wuhle I, II, and Elbe were taken once. 2000 mL water sample were taken from the middle stream and gross filtrated by using glass fiber filters. Each sample was supplemented by 10 g NaCl and concentrated HCl for acidification. RP18 columns were used to concentrate BPA of 1000 mL water sample volume, followed by eluation with acetone. Eluats were dried and resuspended in 1 mL acetonitrile. BPA determination was done by mean of HPLC.

Test Animals

The animals used for the exposure experiments are coming from an animal stock of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany. Adult frogs were induced to spawn by injecting human chorionic gonadotropin (Sigma-Aldrich, Steinheim, Germany) into the dorsal lymph sac according to Kloas et al. (1997). Fertilized eggs and larvae were maintained in 60 L tanks filled with 40 L demineralized water and mixed with 0.25 g/L sea salt (Tropic Marin, Wartenberg, Germany). The water was aerated by using airstones and the water temperature was adjusted to 22 ± 1°C. The pH-value ranged from 7.0 ± 0.5. The light-dark cycle was 12:12 h during the exposure time. Tadpoles were held under these conditions until they reached stages 48 or 51, respectively.

Short-term exposure of stage 51 tadpoles

Determination of potential effects of BPA on thyroid system was accomplished by exposing tadpoles at stage 51 to BPA alone (100, 250, and 500 µg/L) and to BPA plus 0.1 nM T3 (thyroid hormone (TH)) using a semi-static exposure system (Fig. 2) (n=30, respectively). Stage 51 is particularly qualified for this kind of gene expression determination, because the thyroid gland is not yet functioning. Consequently, no endogenous TH circulate in

the blood stream, but several tissues are already competent to respond very sensitively to exogenous addition of TH by modified gene expression patterns. Test solutions were changed daily.
After 24, 48, and 72 h, head tissue was sampled to analyze the thyroid system-specific biomarker thyroid hormone receptor ß (TRß) at the gene expression level.
All test chemicals (E2, BPA, T3) were purchased from Sigma (Taufkirchen, Germany).

Long-term exposure of stage 48 tadpoles

To determine the effects of BPA on morphological parameter (body weight, whole body length), gonadal gross morphology and histology tadpoles at stage 48 were exposed to BPA concentrations ranging from 10^{-9} to 10^{-6} M for up to 75 days using a flow through system (Fig. 2). In parallel the natural estrogen 17ß-estradiol (E2) was used as a positive control at 0.2 µg/L. Each treatment contained 4 tanks with 7 L test solution and 25 tadpoles, respectively. At the end of metamorphosis (stage 66), body weight, whole body length and phenotypic sex was determined for all animals. Gonadal tissues were collected for histological analyses.

Fig. 2: Design of a semistatistic system and a flow through sytem.

Abb. 2: Aufbau eines semistatischen Systems und eines Durchfluss-Systems.

Gene expression determination in head tissues

Total RNA of head tissues was isolated using the phenolic reagent Trizol (Invitrogen, Karlsruhe, Germany). Diluted RNA was transcribed in cDNA by reverse transcription (RT). Following the amplification of cDNA for EF1a, and TRß genes were carried out as described by Jagnytsch et al. (2005).

1.3 Results and Discussion

Bisphenol A emission

The total emission of BPA into the Elbe catchment was found to be 970 kg/a by WWTP, 70 kg/a by sewer systems and 510 kg/a by two industrial discharges in the Czech part of Elbe (Fig. 3). The retention within the surface waters of Elbe is 790 kg/a or 51%
The specific emission of 0.24 g/a*inhabitant was calculated. The inhabitant specific discharge out of sewage plants was calculated to be 0.056 g/a*inhabitant. No data were found to atmospheric deposition, particle entry via erosion, dissolved entry via avulsion, basis flow, interflow, and tile drainage. The calculated specific emission of BPA correspond with data

found in literature (Fries, 2002), which specify a range 0.013-0.144 g/a*inhabitant.

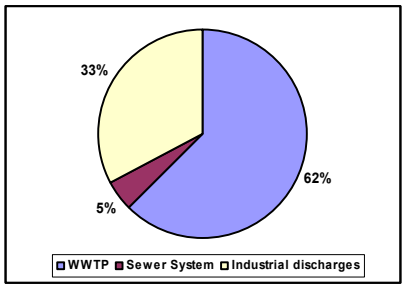

Fig. 3: Total emission of BPA into Elbe catchment.

Abb. 3: Gesamt-Emission von BPA im Elbe-Einzugsgebiet.

Using the retention function several BPA concentrations could be calculated for different monitoring stations among the Elbe river. The comparison of these calculated concentrations versus observed BPA concentrations has shown that most of the data were within the 30% deviation but some data were underestimated especially stations downriver of Valy (Fig. 4A). Possible reason for that could be emssions of industrial discharges like Spolchemie and Spolana in the Czech area. Considering these industrial discharges all values were within the 30% deviation of retention (Fig. 4B).

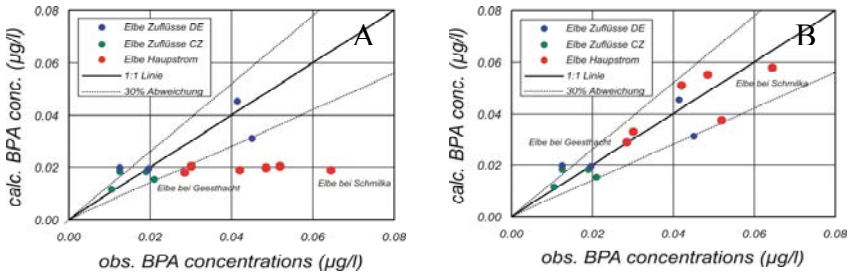

Fig. 4: Comparison of calculated and observed BPA concentrations among the Elbe river. **A** without industrial discharges. **B** with industrial discharges.

Fig. 4: Vergleich zwischen berechneten und tatsächlich beobachteten BPA-Konzentrationen entlang der Elbe. **A** ohne Berücksichtigung industrieller Direkteinleiter und **B** mit industriellen Direkteinleitern.

BPA determination of water samples of Elbe-river catchment

BPA was determined in water samples taken from different tributaries of the Elbe catchment. Data are given in table 1. BPA could be detected in all tested water systems. The concentrations ranged from 0-1.5 µg/L. The highest BPA concentration could be measured in WWTP Münchehofe outflow.

Tab. 1: BPA detection in water samples of the Elbe-river catchment. The unit of the given data is ng/L.

Tab. 1: BPA-Bestimmung in verschiedenen Wasserproben des Elbe-Einzugsgebietes. Die Einheit der angegebenen Werte ist ng/L.

Water system	Oct 06	Nov 06	Dec 06	Jan 07
Spree (Große Tränke)	0	144.3	0	0
Havel (Hennigsdorf)	127.1	302.7	30.7	112.7
Havel/Spree (Potsdam)	363.5	268.9	20.4	43.6
WWTP Outflow Münchehofe	1062.3	347.0	1002.8	1508.1
Erpe + WWTP Münchehofe	911.8	256.2	627.0	n.d.
Elbe (Krippen)	481.9	n.d.	n.d.	n.d.
Wuhle I (Marzahn)	n.d.	137.8	n.d.	n.d.
Wuhle II (Hellersdorf)	n.d.	112.2	n.d.	n.d.
Erpe above WWTP Münchehofe	n.d.	n.d.	n.d.	547.4

Short-term exposure of stage 51 tadpoles

TRß mRNA expression was significantly up-regulated by 0.1 nM T3 already after 24 h and remained elevated over the entire experimental exposure until 72 h. Within 24 hours, T3 treatment caused a 4-fold higher expression of TRß gene in head tissues compared to untreated controls (Fig. 5A). The highest BPA concentration antagonized the T3-induced TRß expression at all sampling points (Fig. 5B). 250 µg/L BPA caused a significant down-regulation of T3 induced TRß expression after 48 h. In the absence of T3, BPA alone did not affect TRß expression.

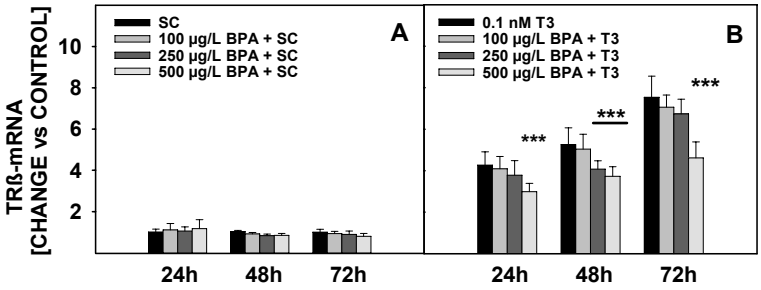

Fig. 5: Relative TRß-mRNA expression after short-term exposure with BPA. **A** BPA compared to untreated control. **B** BPA compared to T3.

Abb. 5: Relative TRß-mRNA Expression nach Kurzzeit-Exposition mit BPA. **A** BPA im Vergleich zur unbehandelten Kontrolle. **B** BPA im Vergleich zur T3-Behandlung.

Long-term exposure of stage 48 tadpoles

At the end of metamorphosis, mean weight of tadpoles treated with BPA was increased in a dose dependent manner being significant already at 10^{-8} M BPA for males and females, respectively (Fig. 6). Mean whole body length of tadpoles exposed to BPA was also increased compared to controls (Fig. 6). This increase was significant at 10^{-6} M BPA for males and at 10^{-7} M and 10^{-6} M BPA for females. At the end of metamorphosis gonads were dissected for histological analyses. The exposure of males to 10^{-6} M BPA caused remarkable changes in gonadal gross morphology. Histological analyses of

these gonads have clearly shown leackages in testicular tissues compared to control animals (Fig. 7A/B). Similar effects could be observed in males which were exposed to the natural estrogen 17ß-estradiol (E2) (Fig. 7C).

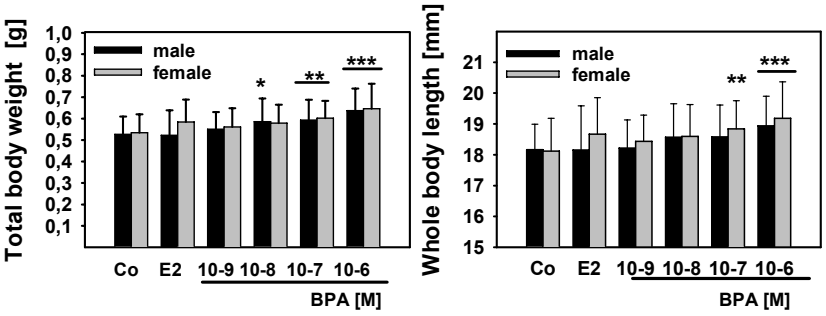

Fig. 6: Total body weight and whole body length after long-term exposure with BPA.

Abb. 6: Gesamtkörpergewicht und Gesamtkörperlänge nach Langzeit-Exposition mit BPA.

At the end of metamorphosis gonads were dissected for histological analyses. The exposure of males to 10-6 M BPA caused remarkable changes in gonadal gross morphology. Histological analyses of these gonads have clearly shown leakages in testicular tissues compared to control animals (Fig. 7A/B). Similar effects could be observed in males exposed to E2 (Fig. 7C).

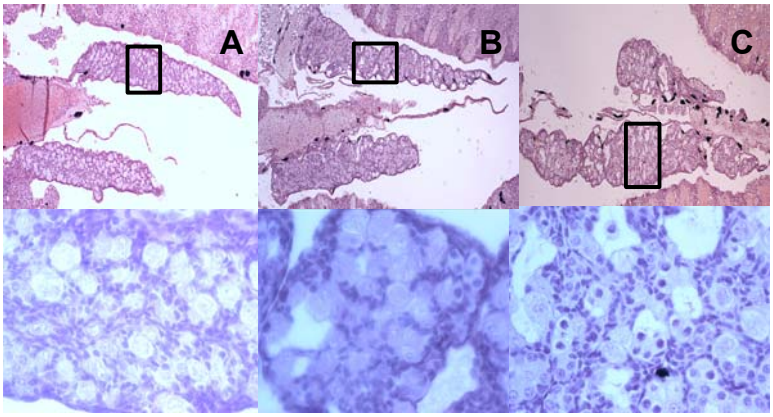

Fig. 7: Hematoxylin-eosin staining of male gonads of juvelile frogs: **A** untreated control male, **B** after 10^{-6} M BPA treatment, **C** after E2 treatment.

Abb. 7: Hämatoxylin-Eosin-Färbung von männlichen Gonaden juveniler Frösche: **A** unbehandelte Kontrolle, **B** nach Behandlung mit 10^{-6} M BPA und **C** nach Behandlung mit E2.

Discussion

Biodegradation experiments have demonstrated a half-life of up to 4 days for BPA in river waters (Klecka et al. 2001). Despite it seems to be degraded

rapidely after a lag phase it could be shown that BPA is permanently detectable in the Elbe River and several tributaries.

Investigations about emissions have shown that mainly WWTP´s and industrial discharges are responsible for the BPA release into the Elbe catchment. A retention rate of 51 % for BPA by WWTP´s was calculated which means the absolute inflow of the WWTP´s must be twice as much. This inflow must be mainly caused by households as well as small and medium sized enterprises. Possible sources of BPA could be toilet paper, plastic material cleaned in a dishwasher or food rests which are disposed by sewer systems (Gehring 2004, EFSA 2006). Another source of BPA is released from PVC pipes or surface coating agents. Depending on state of the technology the retention of WWTP´s are quite different. Old WWTP´s with trickling filtration and activated sludge barely degrade BPA whereas WWTP´s using membrane filtration, nano-filtration and membrane bioreactors have a BPA elimination rate of at least 75% (Gehring 2004).

The amphibian model was used to analyze effects of BPA on the thyroid and reproductive system. Several studies have evaluated that the amphibian *Xenopus laevis* is a valuable model to determine effects of ED on endocrine system (Opitz et al. 2002, Kloas 2002, Levy et al. 2004). TH produced and released from thyroid gland are essential for different developmental phases during metamorphosis. Interferences of ED with thyroid system can be observed by stimulation or deceleration of metamorphosis in long-term exposure.

In this study a short-term exposure with BPA and a combination of TH and BPA was performed to determine effects of BPA on TRß gene expression level. TRß is an early response gene of TH. Within 24 h it responds rapidely to exogenous given TH induction in stage 51 pre-metamorphic tadpoles. Challenge experiment with BPA and T3 clearly demonstrated a dose response inhibitory action of BPA at concentrations of 250 and 500 µg/L BPA on TH induced gene expression of TRß. Similar inhibitory effects have been observed for TBBA which is the brominated form of BPA and a most commonly used flame retardant (Jagnytsch et al. 2006). The BPA effect was significant but less pronounced compared to that caused by TBBA. These results suggest that compounds with similar chemical structure can have similar properties.

Long-term exposure with BPA has shown significant increases in mean body weight and whole body length of juvenile frogs in a dose dependent manner. Studies about effects of BPA on body weight are quite controversial. In several studies it was documented that BPA can cause an increase of single organ weights like uterus and liver in rats and mice (Papaconstantinou et al. 2000). Rubin et al. (2001) has demonstrated the increase of body weight in rat offspring treated by BPA. Further *in vivo* studies have shown a decrease in mouse and chicken testis weight or body weight probably caused by toxic side effects (Al-Hiyasat et al. 2002, Furuya et al. 2002). However, the natural estrogen E2 was also tested in long-term exposure as a positive control and there was no difference in body weight and whole body length seen suggesting that growth promoting effects of BPA are not induced by

estrogenic modes of action but might affect regulation of insulin like growth factors.

Investigations of the reproductive system were done by using long-term exposure where the positive control E2 caused 75% feminization. Remaining E2 males have mixed sex gonads or testis with leakages within the tissue. Exposure of males to 10^{-6} M BPA caused remarkable changes in gonadal gross morphology too. Histological analyses of these gonads also demonstrated leakages in several testicular tissues but less pronounced. From E2 treatment it appeared that feminization of male gonads starts with tissue degradation and disaggregation. After a couple of time testis changes to mixed gonads which means ovarian and testicular tissue in one gonad until just ovarian tissue is remaining. That means remarkable observation in gross morphology and histology of BPA male gonads could be evidences for beginning feminization procedures. So the tested concentrations of BPA were too low for causing complete feminization. In zebrafish 1820 µg/L BPA caused 32% ovo-testis (Yokota et al. 2000).

In summary, BPA action on thyroid system is just moderate resulting in minor competition of BPA and TH on thyroid hormone receptor level or transport binding proteins. Results on gross morphology and histology are not finished yet. But these given results clearly demonstrate that higher BPA concentrations can interfere with the thyroid system probably mediated via its moderate thyroid hormone receptor binding in a competitive manner and also with the reproductive system. Lower concentrations of BPA did not show any remarkable effect. BPA is widely spread in the environment. Especially in regard to animal and human health it is necessary to improve the degradation of such compounds in WWTPs and also to minimize the release of such harmful substances by industries into to surface water.

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