MULTIGENERATIONAL EFFECTS OF THE FLAME RETARDANT TRIS (2-BUTOXYETHYL) PHOSPHATE (TBOEP) IN *DAPHNIA MAGNA*

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Introduction

Tris (2-butoxyethyl) phosphate (TBOEP) is a non-halogenated alternative organophosphorus flame retardant (OPFR) used as a substitute for phased-out brominated flamed retardants (BFRs). Because of its high production volume and its use in a broad range of applications, this chemical is one of the predominant OPFRs found in the environment and it has been detected in air, water and biota [1]. However, limited information is available on the long term effects of TBOEP exposure in aquatic organisms.

In a previous study, we found that sublethal doses of TBOEP altered the transcription of genes related to the biosynthesis as well as the protein and the energy metabolisms of the zooplankton *Daphnia magna* [2]. In the present study, the long-term potential impacts of TBEOP were assessed in this aquatic model species using a multigenerational approach. Pollutants may indeed affect both exposed organisms and their progeny with effects potentially more severe in subsequent generations [3] and offspring can have different tolerance to contaminants as a consequence of parental exposure.

Multigenerational effects of TBEOP were evaluated in *D. magna* over three successive generations at a sublethal concentration of environmental relevance (10 µl/L). Effects were evaluated at the gene, cellular, and individual (i.e., survival, reproduction, and growth) levels. The capacity of offspring to recover after two generations of parental exposure was also investigated.

Materials and methods

Daphnia magna culture and experimental exposure design

Genetically homogenous *D. magna* were cultured in a growth chamber following Environment and Climate Change Canada's method [4].

Chronic exposure to TBOEP over three successive 21d generations (F0, F1 and F2) was performed following OECD guidelines [5] using 17 *D. magna* neonates (<24h) per generation. An additional non-exposed group was added to the F2 generation exposure to monitor the recovery of the offspring after parental exposure (Figure 1). The time elapsed between birth and first brood, the number of offspring, the body size at 21d as well as the number of molts were used as reproductive and growth parameters.

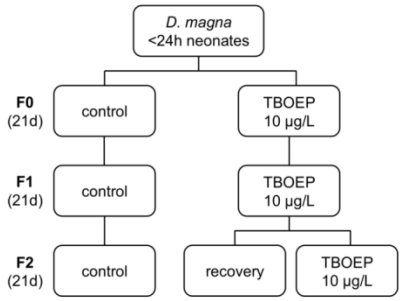

Figure 1. Experimental design for the multigenerational exposure of *D. magna* to TBOEP.

Gene transcription analysis using qRT-PCR

Total RNA was extracted from pools of 2-3 *D. magna* (n=3 replicates per treatment) using RNeasy® plus mini kit (QIAGEN, Canada) following manufacturer instructions. Quantitative real-time PCR (qRT-PCR) analyses were conducted for selected transcripts based on Giraudo et al. [2] and the Comparative Toxicogenomics Database (http://ctdbase.org). Total RNA was reverse transcribed using the QuantiTect® Reverse transcription kit (QIAGEN, Canada) following manufacturer instructions. qRT-PCR analyses were carried out as detailed in Giraudo et al. [2] and expressed as fold change between exposed and control treatments.

Protein biomarkers analysis

Enzyme activities and protein content were measured in pools of 2-3 *D. magna* of whole body homogenates (n=3 replicates per treatment). Catalase activity was measured as described in Dautremepuits et al. [7] and reported in units/min/µg proteins by using a molar extinction coefficient of 0.0436 $mM^{-1}cm^{-1}$. Juvenile hormone esterase (JHE) was measured as previously described in Wheeler et al. [8] and reported as mM α-naphtol/min/mg protein. The immune-specific detection of vitellogenin (VTG) was assessed by Western blotting based on Houde et al. [9] and expressed as the ratios of the Western Blot band OD to the protein concentration (µg/mL). Alpha (α)-amylase (AMY) activity was measured using Abcam Amylase Assay kit (ab102523) according to the manufacturer's instructions and reported as nmol nitrophenol/min/µg protein.

Results and discussion

Life-history endpoints

Multigeneration effects of TBOEP exposure on reproductive and growth parameters are presented in Table 1. The number of neonates per female and body size of adults were not significantly affected by exposure to TBOEP of the current generation, but the number of molts was significantly reduced in the exposed individuals compared to control groups. Exposure of previous generations to the chemical did not affect the molting frequency but may have had an effect on the size of daphnids. Growing a third generation of offspring in a clean medium did not significantly improve reproduction, nor did it affect growth parameters.

Table 1. Multigeneration effects of TBOEP exposure on *D. magna* life-history endpoints.

		F0	F1	F2
Length (mm)	Control	4.35 ± 0.16	4.39 ± 0.23	4.31 ± 0.17
	TBOEP 10µg/L	4.42 ± 0.13	4.16 ± 0.15*	4.21 ± 0.20*
	Recovery			4.18 ± 0.19
Width (mm)	Control	3.09 ± 0.09	3.07 ± 0.02	3.10 ± 0.15
	TBOEP 10µg/L	3.12 ± 0.07	2.91 ± 0.12*	2.97 ± 0.17*
	Recovery			2.97 ± 0.12
Neonates/individual	Control	161 ± 26	106 ± 25**	137 ± 30*
	TBOEP 10µg/L	168 ± 28	112 ± 27	127 ± 23
	Recovery			127 ± 34
Molts/individual	Control	8.6 ± 0.5	8.6 ± 0.5	8.6 ± 0.7
	TBOEP 10µg/L	8.2 ± 0.6#	8.5 ± 0.6#	8.2 ± 0.7#
	recovery			8.6 ± 0.5

* indicates a generation effect as measured by the generalized Conway-Maxwell-Poisson distribution for the number of neonates and by linear regression for the body size (*p<0.05; **p<0.0001)

# indicates an effect of TBOEP exposure regardless of the generation as measured by the generalized Conway-Maxwell-Poisson distribution (#p<0.01)

Effects on gene transcription

TBOEP exposure significantly affected the transcription of genes involved in protein and energy metabolism (*catd*, *acly*) as well as oxygen transport (*dhb1* and *dhb2*)(Figure 2), which confirms our previous results suggesting potential biomarkers of TBOEP exposure [2]. In addition, TBOEP affected genes involved in oxidative stress (*gst*, *cat*) and endocrine-related processes (*ecr*, *jhe*).

Statistical analyses showed that the transcription of the genes affected by TBOEP exposure was not significantly different between generations, whereas a different set of genes showed a difference in transcription only between generations (Figure 3). These genes are involved in reproduction (*vtg1*, *vtg2*), hormone-mediated processes (*hr3*), as well as detoxification (*ahr*) and metabolic processes (*amyl*), suggesting long-term endocrine disruption potential of TBOEP.

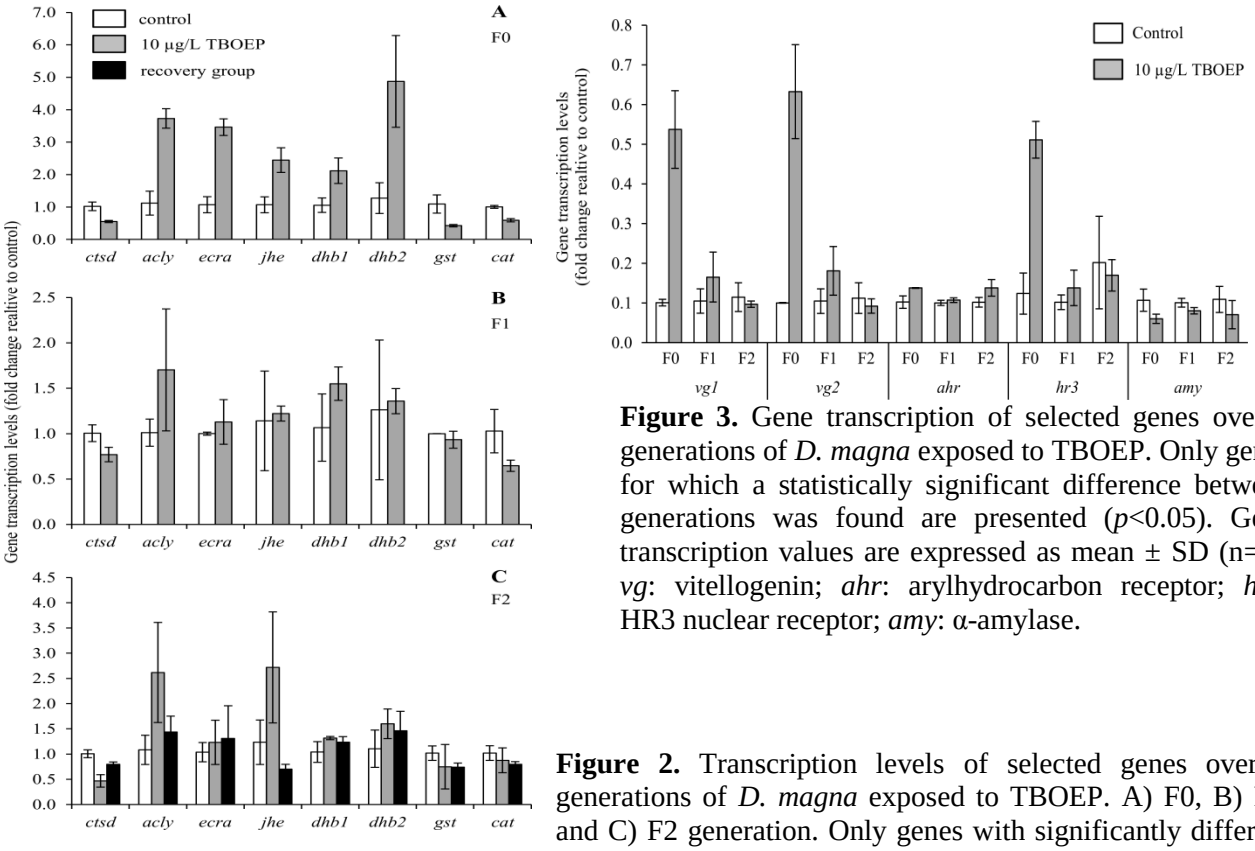

Figure 3. Gene transcription of selected genes over 3 generations of *D. magna* exposed to TBOEP. Only genes for which a statistically significant difference between generations was found are presented (p<0.05). Gene transcription values are expressed as mean ± SD (n=3). *vg*: vitellogenin; *ahr*: arylhydrocarbon receptor; *hr3*: HR3 nuclear receptor; *amy*: α-amylase.

Figure 2. Transcription levels of selected genes over 3 generations of *D. magna* exposed to TBOEP. A) F0, B) F1, and C) F2 generation. Only genes with significantly different transcription levels between treated and control organisms for a given generation are presented (p<0.05). No significant differences were observed between generations. Gene transcription values are expressed as mean ± SD of the fold change (n=3). *ctsd*: cathepsin D; *acly*: ATP-citrate synthase; *ecra*: ecdysone receptor A; *jhe*: juvenile hormone esterase; *dhb*: hemoglobin; *gst*: glutathione-S-transferase; *cat*: catalase.

Protein biomarkers

Transcriptional effects of TBOEP were confirmed at the protein level for VTG and AMY but only JHE showed intergenerational effects (Figure 4).

Altogether, these results suggest that TBOEP can have long-term transmissible multigenerational effects on essential biological processes including endocrine-disruption potential.

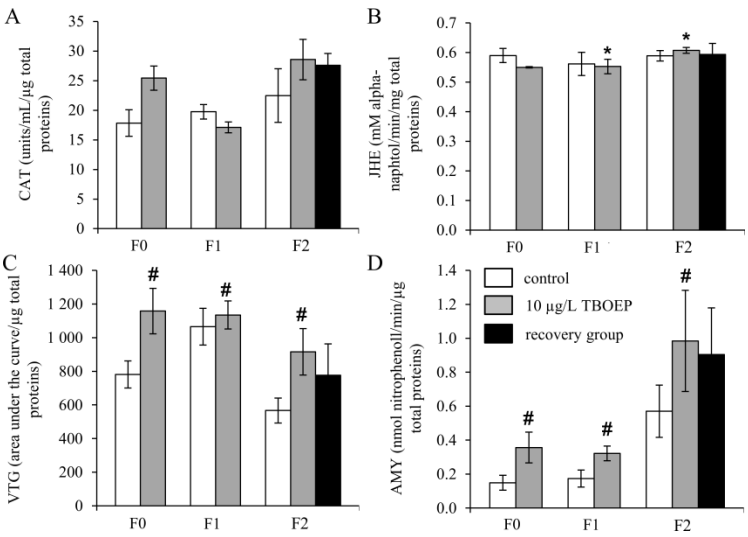

Figure 4. Protein biomarker measurement over 3 generations of *D. magna* exposed to TBOEP. A) CAT, B) JHE, C) VTG and D) AMY. * indicates a generation effect (*p<0.05; Spearman correlation). # indicates an effect of TBOEP exposure regardless of the generation (#p<0.01; van Elteren test).

Acknowledgements

This study was funded by Environment and Climate Change Canada's Chemicals Management Plan (CMP).

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