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No Estrogenic Effects of Bisphenol A in *Daphnia magna* STRAUS

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Studies on the effects of endocrine-disrupting industrial chemicals have centered mainly on effects in humans and on *in-vivo* findings in mammals, reptiles and fish. More detailed information about the effects on and mechanisms of action in invertebrates has only been obtained from a few striking individual cases. The existing literature often glosses over the lack of detailed knowledge about this subject with vague formulations, or attempts to compensate by deriving unsubstantiated generalizations from individual findings. In recent years, this deficit of knowledge on the influence of endocrine disrupters on the reproductive health of invertebrates has been universally deplored (Shurin and Dodson 1997; Campbell and Hutchinson 1998); some research activities are now being started by various scientific, governmental, and industrial organisations.

Daphnia magna STRAUS (Cladocera, Daphnidae), a characteristic and common representative of the freshwater zooplankton, has been used world-wide for many years as a test species for determining the acute and chronic toxicity of industrial chemicals and crop protection products. This species can also be used in the 21d reproduction test (according to OECD 202; latest draft January 1996) to screen for potential adverse effects caused by endocrine-disrupting substances without providing mechanistic information (cf. OECD 1997). More recently, physiological and biochemical processes associated with the application of xeno-estrogens have been studied in this species (Baldwin and LeBlanc 1994; Baldwin et al. 1995, 1997, 1998).

This experimental study is intended to check the potential estrogen-like influence of Bisphenol A on the moulting behaviour of parthenogenetic females of *Daphnia magna.* Moulting behaviour has been claimed to be a toxicological endpoint which is able to reflect effects of endocrine-disrupting chemicals (Baldwin et al. 1995; Zou and Fingerman 1997). The question arises whether this hypothesis is also valid for a substance like Bisphenol A which is readily biodegradable (DOW, internal study), not bioaccumulative, and whose estrogenic potential has been determined

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in-vitro only (low estrogen-receptor binding affinity), with no evidence of specific hormonal influences in mammalian or other vertebrate reproduction tests (cf. GDCh-Advisory Committee on Existing Chemicals of Environmental Relevance 1997).

MATERIALS AND METHODS

The Bisphenol A used in this study (CAS no. 80-05-7; molecular formula $C_{15}H_{16}0_2$; purity: 99.94%) was produced by Bayer; the test sample was provided under GLP conditions.

For more than 10 years, *Daphnia* magna has been kept in Bayer AG's ecotoxicology laboratories as a synchronous parthenogenetic strain at 20°C and at a photo period of 16 hr light and 8 hr darkness. The only food source is the single-cell green algae *Scenedesmus subspicatus* CHODAT from fermenter cultivation (batch operation).

Data on the acute *Daphnia* toxicity of Bisphenol A (toxicological endpoint: immobilization) were taken from the literature (summarized by Staples et al. 1998). In order to complement the data for the current EU risk assessment, the chronic *Daphnia* toxicity (toxicological endpoint: reproduction rate) was determined under GLP conditions in accordance with the OECD Draft Guideline 202 (version dated August 1995) in a semi-static test (medium renewal three times per week) with individual animals in 10 parallels of each concentration step. The test concentrations covered the interval from the lowest 48 hr-EC50 (Stephenson 1983: 3.9 ppm) down to 1/100th of this concentration, following a geometric progression with a factor of 3.16. The results (NOEC/LOEC) are based on the analytically measured values (HPLC: analytical limit of detection: 0.01 ppm) and represent the arithmetic mean of all measured values before and immediately after the renewal of the test media within the semi-static procedure. In a subsequent experiment, the number of pre-adult and adult moults was determined during a 21 d exposure (Bisphenol A: 3.16 or 0.316 ppm; control) in order to obtain a suitable data base for a comparison with the findings and conclusions reached by Baldwin et al. (1995) and Zou and Fingerman (1997).

RESULTS AND DISCUSSION

The data on the acute and chronic *Daphnia* toxicity of Bisphenol A are listed in Table 1. In the 21 d reproduction test, no effects deviating from the control were observed at the highest test concentration (3.16 ppm). This allows us to conclude that chronic effects do not occur in Bisphenol A below the toxicity threshold for acute effects. The retardation of the moults described by Zou and Fingerman (1997) for DES (at $\geq$ 0.1 ppm) and

endosulfan (at ≥ 0.1 ppm) was likewise not observed in this case (cf. Table 2).

Table 1. *Daphnia* toxicity of Bisphenol A.

Acute *Daphnia* toxicity (immobilization)	**48 hr EC_{50} (ppm)**	**Remarks**
Staples et al. (1998)	10 **20** **3.9**	
Chronic *Daphnia* toxicity (reproduction)	**21 d NOEC (ppm)**	**Remarks**
Caspers (1997)	> 3.16	reproduction rate (no. of live offspring/parent animal after 21 d of exposure): control (n = 10): 125.7 ± 8.1 3.16 ppm (n = 10): 124.3 ± 10.6

Table 2. Effects of Bisphenol A on the moulting behavior of parthenogenetic females of *Daphnia magna.*[a]

Treatment (ppm)	**Pre-adult moults (mean ± SD)**	**Total moults within 21 d (mean ± SD)**
control (n = 9)	3.9 ± 0.33	7.9 ± 0.33
0.316 (n = 10)	4.0 ± 0.47	8.0 ± 0.47
3.16 (n = 9)	4.1 ± 0.33	8.1 ± 0.33

[a]None of these figures are significantly different from the control figures at a = 0.05 (Student-*t*-Test).

A critical evaluation of the experimental approach of the authors Zou and Fingerman casts doubt on the validity of their data in any case. Their *Daphnia magna* clone does not enter the reproductive phase before day 14. The cumulative mortality rate in the control group is already 15% at this point in time. The reproduction rate in the control is only approximately 10 young per parent *Daphnia* in 40 days; OECD Draft Guideline 202 requires a reproduction rate of ≥ 60 young per parent *Daphnia* within 21 d as a validity criterion. The OECD Draft Guideline recommends daily

feeding with living algal cells if possible, but Zou and Fingerman only provided dried algae at 3 d intervals.

These weak points may not have been the reason for the observed changes in pre-adult moulting behavior. However, there remains a basic skepticism regarding the fitness of the *Daphnia* used by Zou and Fingerman, and thus regarding their suitability for ecotoxicological test purposes. In any case, an extension of the test period up to 21 d would have been desirable in order to prove the relevance of reduced moulting frequency on the population dynamics of *Daphnia magna.*

Baldwin et al. (1995) observed a prolongation of moulting cycles in young *Daphnia* under the influence of DES (at 0.5 ppm), which however, on a long-term scale, had no negative effect on the reproduction rate until the following generation. On the other hand, the normalization of the moulting cycles reported in this second generation - interpreted by the authors as an “acclimatization effect” - is surprising; at the very least, it raises the question of the causes and mechanisms and also the natural variability and ecotoxicological significance of this signal. Our own investigations on the moulting frequency under constant laboratory conditions (20°C; 16 hr light, 8 hr dark; M4 medium; constant feeding with unicellular green algae according to OECD 202) showed an approximately 20% lower number of moults in 21 d than that stated by Baldwin et al. (1995) (cf. Table 2). It seems that the moulting behavior is an extremely variable factor in *Daphnia magna* without any deeper ecotoxicological significance. Rather it is simply an expression of the ability of *Daphnia magna* to adapt to variable environmental conditions.

In our study, no statistically valid difference in the moulting frequency between the control batch and the two Bisphenol A batches (0.316 ppm; 3.16 ppm) was determined at the significance level $\alpha = 0.05$ (cf. Table 2). Thus, both chronic endpoints, moulting behavior and reproduction of parthenogenetic females, have sensitivities similar to the immobilization rate in the acute test (cf. Table 1). On the other hand, deficiencies in the methods of Zou and Fingerman (1997) cast doubts not only on the validity of their data, but also on the general suitability of the moulting cycle and frequency of *Daphnia magna* as an indicator of xeno-estrogenic effects.

No published records on endocrine-modulating effects of Bisphenol A exist for the aquatic environment. The only *in-vitro* screening test indicating potential endocrine effects of Bisphenol A in fish (Sumpter and Jobling 1995) revealed no effects below the threshold of traditional ecotoxicological endpoints.

An overview of the existing ecotoxicological data for different trophic levels of aquatic life has been presented by Staples et al. (1998).

Table 3. Aquatic toxicity data of Bisphenol A (drawn from Staples et al. 1998)

Organism	Toxicological endpoint	Results
Microorganisms	growth inhibition	IC_{50}: 54.5 ppm
Freshwater green algae	cell count cell volume	96 hr EC_{50}: 2.5 - 3.1 ppm 96 hr NOEC: 1.17 ppm
Freshwater invertebrates	*Daphnia magna* (see Table 1)	
Freshwater fish	mortality	48 hr - 96 hr LC_{50}: 3.0 - 15.0 ppm

These data (and ongoing studies on growth inhibition in fish; Bayer Company, not yet finalized) lead us to suppose that there are only slight, if any, chronic effects of Bisphenol A on aquatic organisms below the limit of acute effects.

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