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Environment International 28 (2003) 633–638
ENVIRONMENT INTERNATIONAL
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Effects of 17α-ethinylestradiol on the reproduction of the cladoceran species *Ceriodaphnia reticulata* and *Sida crystallina*

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Abstract

Single-species tests allow the assessment of chronical effects of endocrine disruptors on organisms under laboratory conditions. In the current study, three-generation tests with *Ceriodaphnia reticulata* and *Sida crystallina* were carried out to examine the influence of the synthetic hormone 17α-ethinylestradiol (EE) on the reproduction of these cladoceran species. For each species, six different concentrations (10–500 μg/l EE) and two controls were tested with eight replicates for a duration of 4 weeks. The test was initiated by transferring one neonate individual into a test vessel which was incubated under standardized conditions. Every 2 days, the medium was renewed and life history parameters such as survivorship of the adults and juveniles, clutch size, first appearance and number of produced offspring were investigated. Acute toxicity tests showed that *C. reticulata* (EC50 (24 h) 1814 μg/l) was more sensitive towards the substance compared to *S. crystallina* (EC50 (24 h) >4100 μg/l). The juvenile phase of *S. crystallina* was significantly shorter at concentrations above 100 μg/l EE. For *C. reticulata*, 17α-ethinylestradiol caused a higher mortality of the newly hatched juveniles at EE concentrations above 200 μg/l. No effects were found for mortality of adult animals, birth rate, number of juveniles per female and net reproduction rate of *S. crystallina* and *C. reticulata*. Thus, sublethal effects on parental generation exposed to EE lead to disturbances in reproduction and to affection of their offspring. Negative consequences for the population dynamic cannot be excluded, e.g. the decrease of a population.

Keywords: Endocrine disruptor; Synthetic hormones; Ethinylestradiol; Cladocerans; Reproductive effects; Acute and chronic toxicity

1. Introduction

Environmental endocrine disruptors are known as substances that may modify the normal functioning of human and wildlife endocrine, or hormone, system. These exogenous agents interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body. They may cause a variety of problems in terms of development, behavior and reproduction and have the potential to impact both human and wildlife populations (US EPA, 1997; NAS, 1999; Colborn et al., 1993). Chemical and technical products, which mimic the female sex hormone 17β-estradiol, are present in the environment. Some of them enter surface waters through effluents of wastewater treatment plants where they are degraded incompletely. Ternes et al. (1999) found elimination rates for estrogens of 68% (16α-hydroxyestrone) and about 64% (17β-estradiol) in German municipal sewage treatment plants. Notable reduction of estrone and 17α-ethinylestradiol loads while passing through the wastewater treatment plant could not be detected. Various studies showed the hormonal effects of such substances on mammals (e.g. decreasing sperm quality, Carlsen et al., 1992), reptiles (e.g. alligators, Vonier et al., 1996), amphibians (e.g. Tiger Salamanders, Clark et al., 1997), fish (e.g. rainbow trout, Jobling et al., 1995) and some invertebrates (e.g. mollusks, Matthiessen and Gibbs, 1997). The main component of the contraceptive pill, the synthetic hormone 17α-ethinylestradiol (EE), is an endocrine disruptor with estrogenic property. Concentrations of 17α-ethinylestradiol in the environment were determined in the lower ng/l range (Stumpf et al., 1996). Various results were found in different

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0160-4120/02/$ - see front matter
PII: S0160-4120(02)00101-0

toxicity tests with EE. In a test with *Vibrio fischeri* regarding to the effects on the bioluminescence, an EC50 of 212 mg/l was calculated. For *Daphnia magna*, an EC50 (24 h) of 5.7 mg/l could be determined (Kopf, 1997). The reproduction of the rotifer species *Brachionus calyciflorus* was affected at EE concentrations >201 μg/l (Radix et al., 2002). Effects at considerably lower concentrations were observed in a *Scenedesmus* growth inhibition test (EC50 (72h): 0.84 mg/l) (Kopf, 1997) and in a yeast assay (2.2 μg/l) (Rehmann et al., 1999). The effect concentrations of EE on the endocrine system of rainbow trout (*Onchorhynchus mykiss*) were >2 ng/l (Jobling and Sumpter, 1993). In the current study, single-species tests were conducted to assess acute toxicity and also chronic effects of endocrine disruptors under laboratory conditions. Three-generation tests with *Ceriodaphnia reticulata* and *Sida crystallina* were carried out to examine the influence of the synthetic hormone 17α-ethinylestradiol on the reproduction of these cladoceran species.

2. Methods and materials

2.1. Materials

Ethinylestreadiol: 19-Nor-17α-pregna-1,3,5(10)-trien-20-in-3,17-diol[17α-Ethinyl-1,3,5(10)-östra-trien-3,17β-diol], $C_{20}H_{24}O_2$, purity minimum 98%, Sigma-Aldrich, D-Deisenhofen.
Potassium dichromate: purity minimum 99.9% Merck, D-Darmstadt.
Climate chamber: Heraeus Vötsch Heraphyt Zelle, Vötsch Industrietechnik, D-Bahlingen-Frommern. Typ: HPZ 90/50/S.
Photometer: Cary 3, Varian, D-Darmstadt.
HPLC: Advanced LC Sample Processor ISS 200, Diode Array Detector 235C, Perkin Elmer, D-Überlingen.

2.2. Breeding of test organisms

The test species *C. reticulata* and *S. crystallina* were isolated from outdoor zooplankton samples and were kept in 2-l beakers filled with autoclaved water (121 °C, 60 min) from lake Ammersee (Bavaria, southern Germany). Every 2 days, the animals were fed with *Monoraphidium minutum*. The water was renewed in weekly intervals.

2.3. Preparation of the EE stock solution

About 3 to 4 mg EE was dissolved in 1 l of water (autoclaved (121 °C, 60 min) and filtered) stirred with a magnetic stirrer at 500 rpm for 7 to 10 days. Remaining crystals were removed by filtration of the solution with folded cellulose filters for qualitative analysis (Schleicher and Schuell). The EE concentration of the stock solution was measured by HPLC and respectively by UV spectrometry by detecting an absorption spectrum from 200 to 300 nm with maxima at 220 resp. 280 nm. Concentrations in the stock solutions were calculated in relation to a calibration curve of known EE concentrations.

Table 1
Nominal concentrations of EE in the acute toxicity tests with *Ceriodaphnia reticulata* and *Sida crystallina*

Concentration	1	2	3	4	5	6	7	8
EE (μg/l)	410	656	984	1640	2050	2460	3280	4100

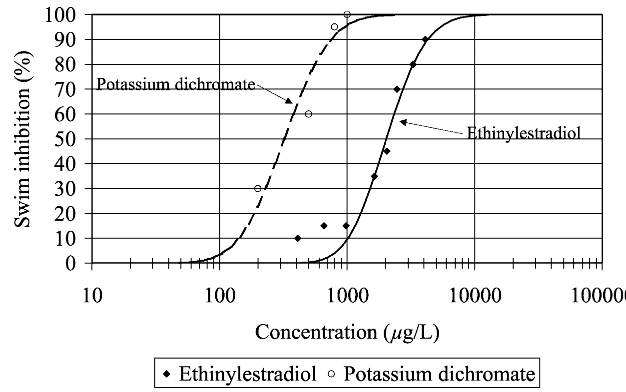

Fig. 1. Dose–responsive curves for 17α-ethinylestradiol and potassium dichromate concentrations (average of two replicates) versus acute toxicity in *C. reticulata*.

2.4. Acute toxicity tests

To specify the acute toxicity of EE for *C. reticulata* and *S. crystallina*, test organisms were exposed in 50-ml test vessels in a climate chamber under standardized conditions (52–56% humidity, 16 h light (25 °C), 8 h (22 °C) darkness). Two replicates with eight concentrations (Table 1) and one control were tested. To indicate possible disturbances of the test process, potassium dichromate with known swim-inhibiting effect on cladoceran is served as positive control substance in four different concentrations (0.2 to 1.0 mg/l). The swim inhibition was observed after 24 h for 10 juvenile cladocerans per concentration. EC10, EC50 and EC90 values were calculated with the Probit model from the concentration–response curves fitted by a sigmoidal function (Fig. 1).

2.5. Chronic toxicity tests

Six different concentrations (10–500 μg/l EE, A–F, eight replicates each) based on the calculated EC values and one control (K, 16 replicates) for each species were exposed for a period of 4 weeks to investigate chronic effects of EE in the three-generation tests (Table 2).

The test was initiated by transferring one neonate individual into a test vessel, which was incubated under standardized conditions (52–56% humidity, 16 h light (25 °C), 8 h (22 °C) darkness). Every 2 days, the medium was renewed

Table 2
Nominal concentrations of EE in the chronic toxicity tests with *Ceriodaphnia reticulata* and *Sida crystallina*

Concentration	K	A	B	C	D	E	F
Ceriodaphnia reticulata							
EE (μg/l)	0	10	50	80	100	200	300
Sida crystallina							
EE (μg/l)	0	20	50	100	200	350	500

and the life history parameters survivorship of the adults and juveniles, clutch size, first appearance and number of produced offspring were investigated. All juveniles were exposed for additional 48 h at the same concentration, and the number of immobilized individuals was counted.

2.6. Data analysis

ANOVA was applied to assess whether there are statistically significant differences in the concentration-dependent dose groups and the controls ($p = 0.0009$). The differences between Kaplan-Meier survival curves were tested according to Wilcoxon and Wilcox using the statistical program package SAS (SAS Institute, Cary, NC, USA). The correlation coefficient of the survivorship of the juveniles (expressed as percentages) and the concentrations was calculated by linear regression analysis. All statistical evaluations were done at the 95% confidence level.

Table 3
Effective concentrations (24 h, swim inhibition) 17α-ethinylestradiol (EE) on *Ceriodaphnia reticulata* and *Sida crystallina*

	EE	Potassium dichromate
Ceriodaphnia reticulata		
1st Replicate (μg/l)		
EC 10	633	100
EC 50	1731	400
EC 90	4727	900
2nd Replicate (μg/l)		
EC 10	579	100
EC 50	1897	300
EC 90	6218	500
Mean (μg/l)		
EC 10	606	100
EC 50	1814	350
EC 90	5473	700
Sida crystallina		
1st Replicate (μg/l)		
EC 10	>4100	170
EC 50	>4100	580
EC 90	>4100	1983
2nd Replicate (μg/l)		
EC 10	>4100	53
EC 50	>4100	389
EC 90	>4100	2839
Mean (μg/l)		
EC 10	>4100	111
EC 50	>4100	484
EC 90	>4100	2411

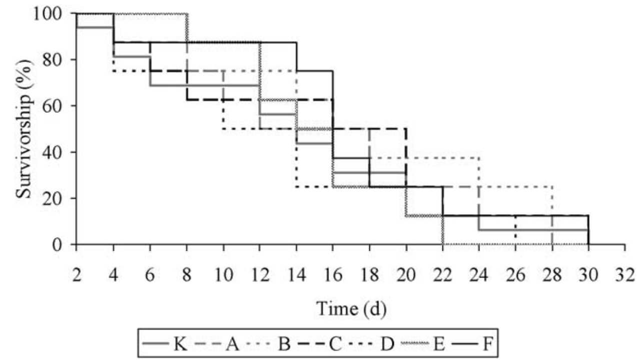

Fig. 2. Survivorship of the adults of *C. reticulata* treated with 17α-ethinylestradiol (0–300 μg/l) for 32 days.

3. Results

3.1. Acute toxicity tests

For *C. reticulata*, an EC50 (24 h) of 1814 μg/l for EE was calculated as mean of two tests (Fig. 1). For *S. crystallina*, the EC50 (24 h) was >4100 μg/l (Table 3). The maximal concentration of the test medium was limited by the solubility of EE in water without a solvent additive.

3.2. Chronic toxicity tests

3.2.1. Mortality of the adults of C. reticulata and S. crystallina

At the beginning of the experiments, the number of test organisms for each species was 16 in the controls and 8 in each of the EE concentrations. The survivorship in the test with *C. reticulata* was 50% until day 12 and day 16 respectively for all groups and reached 0% after 22 to 30 days (Fig. 2).

In the study with *S. crystallina*, nearly 60% of the animals survived in all groups until the test was terminated

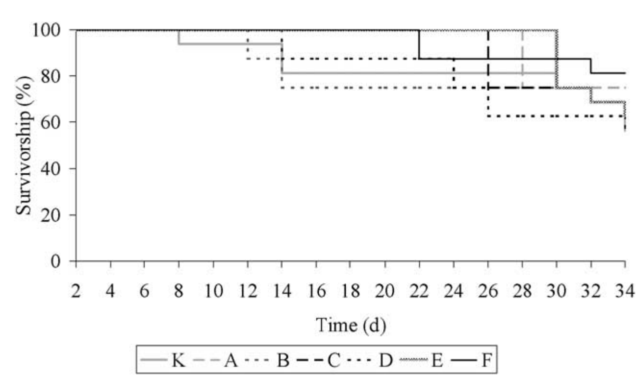

Fig. 3. Survivorship of the adults of *S. crystallina* treated with 17α-ethinylestradiol (0–500 μg/l) for 34 days.

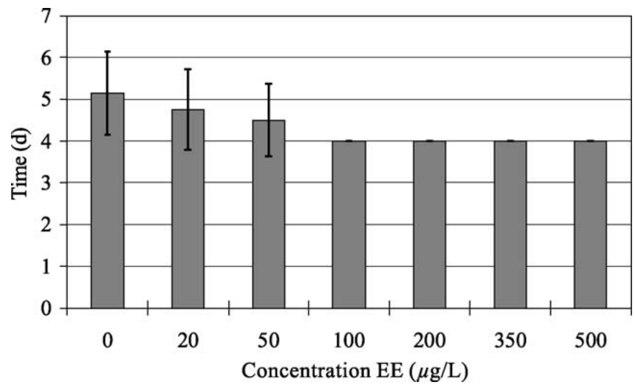

Fig. 4. Length of juvenile phase of *S. crystallina* at different concentrations of 17α-ethinylestradiol.

on day 34 (Fig. 3). The highest survivorship was found for the highest concentration (F, 500 μg/l) with 100% (day 20) and 81% on day 34. In the controls (K) and group D (200 μg/l), at the end of the test, 56% of the test animals survived. At a test concentration of 50 μg/l (B), the first animals died between day 6 and 12 and the survivorship decreased to 69%. The survivorship of groups C (100 μg/l), A (20 μg/l) and E (350 μg/l) was ranged from 21% to 38%; the first dead animals occurred found on day 22.

The statistical evaluations (Kaplan-Meier, Wilcoxon) did not unveil any significant difference between the treated groups (A–F) and the controls.

3.2.2. Length of the juvenile phase of the adults

The juvenile phase is defined as the period from the birth of an animal to the first production of eggs. The average length of the juvenile phase per group was calculated. In the controls and at concentrations up to 50 μg/l, the animals produce first eggs between 4 and 6 days (mean: 5.1, resp. 4.8, resp. 4.5 days). With a duration of exactly 4 days, the juvenile phase of *S. crystallina* (Fig. 4) was significantly shorter ($p = 0.0009$) for each test animal at concentrations above 100 μg/l EE compared to the controls (5 days).

In contrast to this, there were no comparable findings for *C. reticulata* (Fig. 5). In the controls and the treated groups

Table 4
Length of reproduction phase and mean of generated juveniles per female of *Ceriodaphnia reticulata* treated with different concentrations of 17α-ethinylestradiol (0–300 μg/l)

	K	A	B	C	D	E	F
EE (μg/l)	0	10	50	80	100	200	300
Length of maximal reproduction (no. of considered females ≥ 3	12	12	16	14	8	8	8
Length of reproduction (no. of considered females ≥ 1	24	14	24	22	8	8	8
Mean value of generated juveniles per female while max. reproduction	8.1	7.4	11.7	4.9	7.8	6.0	3.4

A (10 μg/l) and B (50 μg/l), the length of the juvenile phase ranged from 4.0 to 4.3 days and for C (80 μg/l) from 4.8 to 6.5 days. The animals in the groups treated with 100 μg/l (D), 200 μg/l (E) and 300 μg/l (F) produced the first eggs after 5 days. Significant differences between controls and EE-treated groups could not be found.

3.2.3. Length of the reproduction phase of the adults

The reproduction phase is defined as the period of time in which juveniles are produced. For the maximal reproduction phase, the period of reproduction is viewed in that at least three females of a group were alive (Table 4).

For *C. reticulata*, juveniles were found from day 4 on in the controls as well as in the treated groups. In the controls and the three lowest concentrations, the length of reproduction phase was 14 for A and 24 for K and B, while the females in the three highest EE-treated groups (D, E and F) produced juveniles for a duration of 8 days. A similar effect for *S. crystallina* could not be found. For this species, the reproduction phase in all groups lasted more than 32 days and was still not completed at the end of the experiment (Table 4).

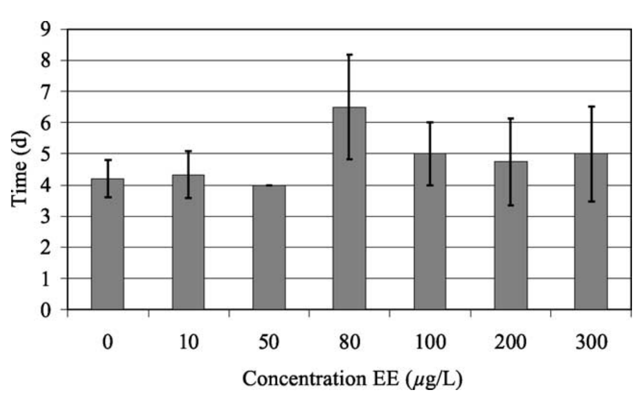

Fig. 5. Length of juvenile phase of *C. reticulata* at different concentrations of 17α-ethinylestradiol.

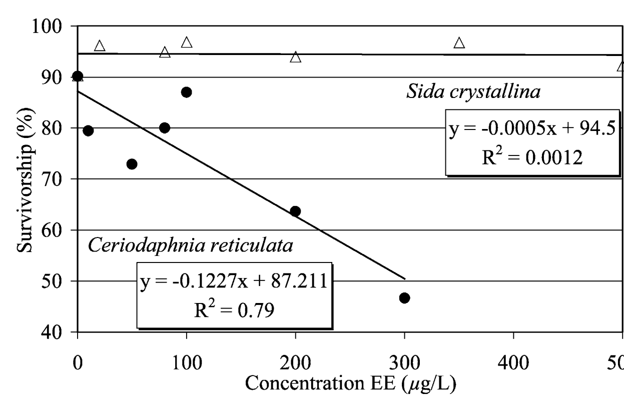

Fig. 6. Survivorship of newly hatched juveniles of *C. reticulata* and *S. crystallina*.

3.2.4. Survivorship of newly hatched juveniles

At EE concentrations above 200 μg/l, a significantly lower survivorship of the newly hatched juveniles of *C. reticulata* (Fig. 6) was found. While in the controls the average of surviving juveniles was more than 90%, at concentrations above 300 μg/l, more than 50% died in the first 48 h after hatching. The survivorship for the juveniles of *S. crystallina* after 48 h was more than 90% for the controls as well as for the treated groups (Fig. 6).

4. Discussion

Aim of the study was to show acute and chronic effects of 17α-ethinylestradiol on *C. reticulata* and *S. crystallina*. In contrast to standardized single-species tests in which *D. magna* bred in laboratory cultures is used as test organism, we isolated *C. reticulata* and *S. crystallina* from natural ecosystems. This might cause a higher sensitivity to chemicals, which has to be considered when data from laboratory tests with single species are transferred to natural biocenosis for statements on effects of substances on population or community level.

Acute toxicity tests demonstrated that *C. reticulata* (EC50 (24 h): 1814 μg/l) was more sensitive towards 17α-ethinylestradiol and also the reference substance potassium dichromate in comparison to *S. crystallina* (EC50 (24 h): >4100 μg/l). For *D. magna*, an EC50 (24 h) of 5.7 mg/l can be found in the literature (Kopf, 1997), indicating a lower sensitivity to the substance.

In the chronic toxicity tests, EE had no effect on the survivorship of the adults of *S. crystallina*. The duration of the test limited recording of the survivorship data to 34 days. In the chronic tests, the concentrations of the treated groups (*C. reticulata*: up to 300 μg/l; *S. crystallina*: up to 500 μg/l) were chosen in the sublethal range, based on the calculated EC50 values. Therefore, effects on the survivorship of the adults were not expected.

A shorter juvenile phase of *S. crystallina* could be shown at concentrations above 100 μg/l. This is possibly induced by a stimulating effect of EE on the egg production or a reaction on stress to ensure the survival of the species by producing offspring as early in their life cycle as possible as it was shown in a study on the effects of xenobiotica on cladocerans (Chu et al., 1997).

In the experiments on the chronic toxicity of EE on *C. reticulata*, a higher mortality of the adults could not be shown during the time of observation.

The exposition with EE resulted in no significant effect on the length of the juvenile phase (between 4 and 6 days) for *C. reticulata*, but treatment with EE in concentrations above 200 μg/l leads to a shorter duration of the reproduction phase. Considering the correlation between these data and the survivorship of the adults, only the time slot, in which at least three adult females of each group were alive, was used for the evaluation. The animals treated with concentrations up to 150 μg EE/l nevertheless generated juveniles for more than 12 days in contrast to the three highest concentrations where the length of reproduction was only 8 days. The exposition with EE concentrations above 100 μg/l might have resulted in a decreased fitness of adult females. Also, a direct hormonal effect of EE on the production of eggs is possible. For the total number of hatched juveniles per female, a significant difference could not be calculated.

EE concentrations above 200 μg/l lead to a lower survivorship of *C. reticulata* in the first 48 h after hatching. The impact of synthetic hormones during the development of juveniles in the females potentially caused physiological defects, lower fitness and also higher sensibility to hormones after birth. A lower survivorship of the larvae of individuals of the fish *Stenotomus chrysops* that were exposed to the endocrine disruptor tributyltin during the embryonic development was shown (Fent and Stegeman, 1991). Also, for amphibians, reptiles and birds, the high sensitivity to environmental toxicants especially in early life stages is known (Fent and Meier, 1994).

The shorter juvenile phase of the adults of *S. crystallina*, the decreased survivorship of newly hatched juveniles and the reduced length of the reproduction phase of *C. reticulata* in EE-treated groups indicate sublethal effects on the parental generation. As a consequence, the reproduction is disturbed and/or juveniles are affected which results in a lower chance of survival of the offspring. This might be a potential risk for the continuity of a species in an ecosystem. By interspecific competition, a slight disturbance of the reproduction like, e.g. a lower survivorship of juveniles, can cause the decrease of a population. Changes in the food web also determine effects on higher trophic levels. Single-species tests are suitable to obtain screening data, but for comprehensive risk assessment multispecies tests, including different trophic levels, as it is realised in microcosm or mesocosm studies, these are inevitable.

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