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27/2015

Statistical Analysis of a Laboratory Study about the Effects of Bisphenol A on the Reproduction of the Ramshorn snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae)

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Umwelt Bundesamt

TEXTE 27/2015

Environmental Research of the
Federal Ministry for the
Environment, Nature Conservation,
Building and Nuclear Safety

Project No. (FKZ) 363 01 245
Report No. (UBA-FB) 002083/E

Statistical Analysis of a Laboratory Study about the Effects of Bisphenol A on the Reproduction oft he Ramshorn snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae)

by

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On behalf of the Federal Environment Agency (Germany)

Imprint

Publisher:
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Wörlitzer Platz 1
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Study performed by:
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Study completed in:
December 2009

Edited by:
Section IV 2.3 Chemicals
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Publication as pdf:
http://www.umweltbundesamt.de/publikationen/statistical-analysis-of-a-laboratory-study-about

ISSN 1862-4804

Dessau-Roßlau, March 2015

The Project underlying this report was supported with funding from the Federal Ministry for the Environment, Nature Conservation, Building and Nuclear safety under project number FKZ 363 01 245. The responsibility for the content of this publication lies with the author(s).

Statistical Analysis of a Laboratory Study About the Effects of Bisphenol A on the Reproduction of the Ramshorn snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae)

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Summary

This report provides the results of a statistical analysis of an experiment with Marisa cornuarietis (Prosobranchia), the subtropical Ramshorn snail, which was performed during the EU Project “Comprendo”. This project aimed at studying the possible impact of endocrine disrupters mainly on invertebrate species. Exposure experiments with Bisphenol A (BPA) were performed as semistatic renewal systems under food surplus. Two replicate groups of 30 sexually mature snails were exposed to nominal BPA concentrations of 0, 0.25, 0.5, 1, and 5 µg/L and a solvent control for 5 months all surviving animals were analyzed with respect to sex and superfemale attributes at the end. BPA was analysed at month 1 of experiment (8 samples over the one day). The following observed variables were provided by the sponsor and J. Oehlmann: survival over time, sex ratio at study end, number of superfemales at study end, the cumulative number of eggs (CNE), the cumulative number of clutches (CNC), and the number of eggs per clutch (NEC). From these variables were calculated: the cumulative number of eggs per female alive (CNEF), the cumulative number of clutches per female alive (CNCF), and the egg and clutch reproduction rates (ERR and CRR, respectively).

The BPA concentration was found to decrease rapidly in the experimental vessels, which does not allow basing the risk assessment on nominal concentrations. Therefore, time-weighted average concentrations (TWA) were calculated but their use gives also reasons for uncertainty. They might be closer to the internal effective concentrations but these remain yet unknown.

There are strong hints that the induction of superfemales (NOEC 0,25 (0,028 TWA) µg/L) and mortality was evoked by BPA. The sex ratio was not visibly affected. Among the reproduction parameters the clutch reproduction rate (CRR) and the egg reproduction rate (ERR) proved to be the variables of choice to compute time-independent toxicity parameters (NOEC, ECx). The statistical design of the study did not allow to determine NOECs (rare exceptions were found but their meaning was unclear; probably statistical artefacts). The EC10, EC20 and EC50 could only be computed using a raw estimation of the concentration/response between solvent control and the 0,25 µg/L BPA-treatment by linear interpolation.

Having in mind the methodological problems and the associated uncertainties, the clutch reproduction rate (CRR) with an EC10 of 0,038 (0,0053 TWA) µg/L BPA was the most sensitive variable, followed by the cumulative number of eggs per female (CNEF) with an EC10 of 0.039 (0,0055 TWA) µg/L BPA and the egg reproduction rate (ERR) with an EC10 of 0,43 (0,0059 TWA) µg/L BPA. Relative to the estimated solvent-control reproduction rates during the main spawning season the rates found here under presence of BPA reflect an induction of less than 10%.

It is strongly recommended for the future, to shorten the experimental time in favour of a better statistical design and the measurement of the reproduction rates, which lead to consistent, time-independent values of the NOEC and ECx, provided the concentration range is chosen appropriately.

Introduction

This report provides the results of a statistical analysis of an experiment with *Marisa cornuarietis* (Prosobranchia), the subtropical Ramshorn snail, which was performed during the EU Project "Comprendo". This project aimed at studying the possible impact of endocrine disrupters mainly on invertebrate species.

The details about the source and breeding of the snails can be found in Oehlmann et al. (2006), who report that exposure experiments with Bisphenol A (BPA, Merck Schuchardt, Hohenbrunn, Germany) were performed as 24-hr (weekends, 48-hr) semistatic renewal systems in 60-L glass aquaria (water volume, 54 L). Animals were fed regularly with TetraMin (Tetra, Melle, Germany) ad libitum. The test under consideration was performed at constant temperature of 20 °C with an equal light:dark cycle (12:12 hr; for the control of the water parameters see Oehlman et al. 2006). The experiment was conducted using an exposure to different nominal concentrations and including a solvent control (SC, ethanol concentration: 12.5 µL/L) and a positive control. The latter is not considered in the present report. Two replicate groups of 30 sexually mature snails (shell height > 20 mm, age > 18 months), each, were exposed to nominal BPA concentrations of 0, 0.25, 0.5, 1, and 5 µg/L for 5 months (February to July) in fully reconstituted water. Animals were acclimatized to exposure temperature 2 weeks before the start of the experiment. Thirty specimens were analyzed at the beginning, and all surviving animals were analyzed at the end. Initial density in the tank was 1.11 snails/L, which dropped to 0.72–1.00 snails/L at the end of the experiment, depending on the mortality in the different groups.

Further, the authors report that BPA was analysed at month 1 of experiment and sampling began 15 min before the change of exposure media and ended 1 day later, before media were changed again (8 Samples). Details of the analytical procedure are reported in Oehlmann et al. (2006). As biological variables the mortality, numbers of eggs, clutches, and eggs per clutch in the tanks were recorded daily (except for weekends). At the end of the experiment snails were narcotized, their shells were cracked and removed. The extensions of all sex organs were measured to the nearest 0.1 mm under a dissection microscope, and malformations such as oviduct ruptures or excrescences on genital and other organs were recorded.

The following observed variables were provided by the sponsor and J. Oehlmann:

- Survival over time
- Sex ratio at study end
- Number of superfemales at study end
- The cumulative number of eggs (CNE)
- The cumulative number of clutches (CNC)
- The number of eggs per clutch (NEC)

The sponsor was interested in a new, independed statistical analysis of the results. To provide this shall be the aim of the present report.

In so doing, at first the following additional variables were calculated from the CNE and CNC for statistical analysis:

- The cumulative number of eggs per female alive (CNEF)
- The cumulative number of clutches per female alive (CNCF)

The CNEF and CNCF depend on the number of females present at a considered day, their reproduction rate [eggs/day] and the time from the start of the experiment until that day. For several reasons the statistical analysis of possible BPA effects will be expanded on the egg and clutch reproduction rates (ERR [eggs/female/day]) and CRR [clutches/female/day], respectively) , because

- the reproduction rate is directly proportional to the metabolic rate and thus to it's possible promotion or inhibition by BPA action;
- under constant environmental conditions (here 20°C; surplus of food) the reproduction rate is constant and independent of time, thus leads to unequivocal values of the NOEC and ECx.

Test Endpoints and Data Analysis

Reproduction

Computation of Additional Variables

In order to calculate CNEF and CNCF the cumulative number of eggs and clutches has to be divided by the number of females present at a considered day. This requires constructing a time dependent survival relationship. At a day when one snail or more than one snail died the number was subtracted from the number of snails present at that day. An example calculation is given in Annex Table 1 (for mortality observations see Annex Table 2) and the summary of establishing time dependent survival data is given by the survival curves presented in Figure 1.

The next step was to estimate the number of survived females. Since the sex of snails cannot be determined at time of introduction into the test aquaria and was not determined by samples of snails during the experimental period, the sex ratio at the experimental end - obtained after dissection of the survived snails - was used as an estimate of number of females alive during each day of the experimental period (Eq. 1).

Eq. 1

$$nfdi = ndi * pf_{lastday}$$

With nfdi: number of females alive at day i
ndi: total number of snails alive at day i
$pf_{lastday}$: proportion of females at the last experimental day (Table 24)

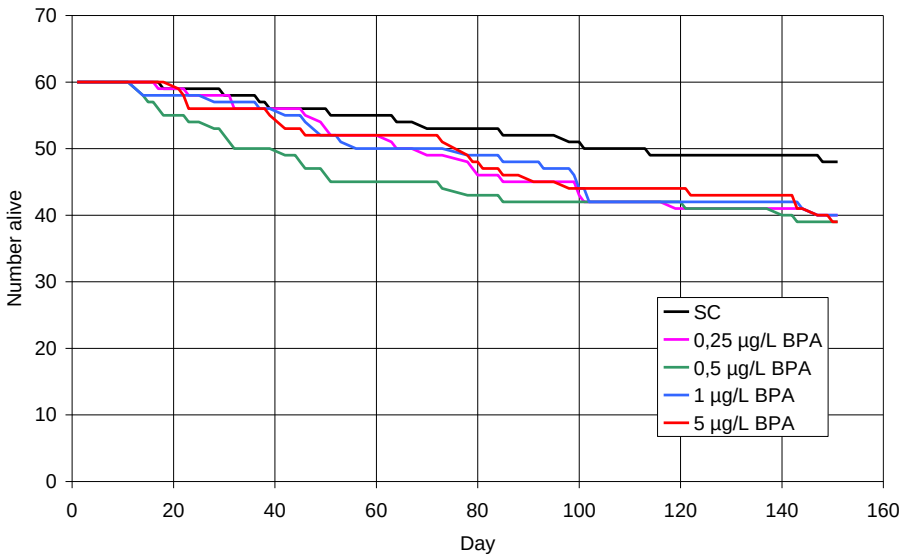

Figure 1 : Survival curves of snails exposed to varius concentrations of BPA as observed during the experimental period (mean of two replicates, each); SC: solvent control.

Since the proportion of females at study end was in most cases higher than 0,5, this estimation appeared more realistic than assuming an 1:1 sex ratio as did Oehlmann et al. (2006). As a consequence, the number of eggs and clutches per female (CNEF, CNCF), as being calculated using Eq. 2 and Eq. 3, are somewhat lower than that calculated by Oelmann et al. (2006). The example calculation is given in Annex Table 2.

Eq. 2

$$CNEF = CNE / nfdi$$

Eq. 3

$$CNCF = CNC / nfdi$$

As an example, the CNE and CNEF curves are shown in Figure 2. The reproduction rates RRE and RRC are equal to the slope of the CNEF and CNCF trend lines (see Figure 2; bottom part). A comparison of the coefficients of determination, R^2, between trend lines for the CNE and CNEF revealed that more of the variance war explained in case of the CNEF, shown by higher values of R^2 (Table 1).

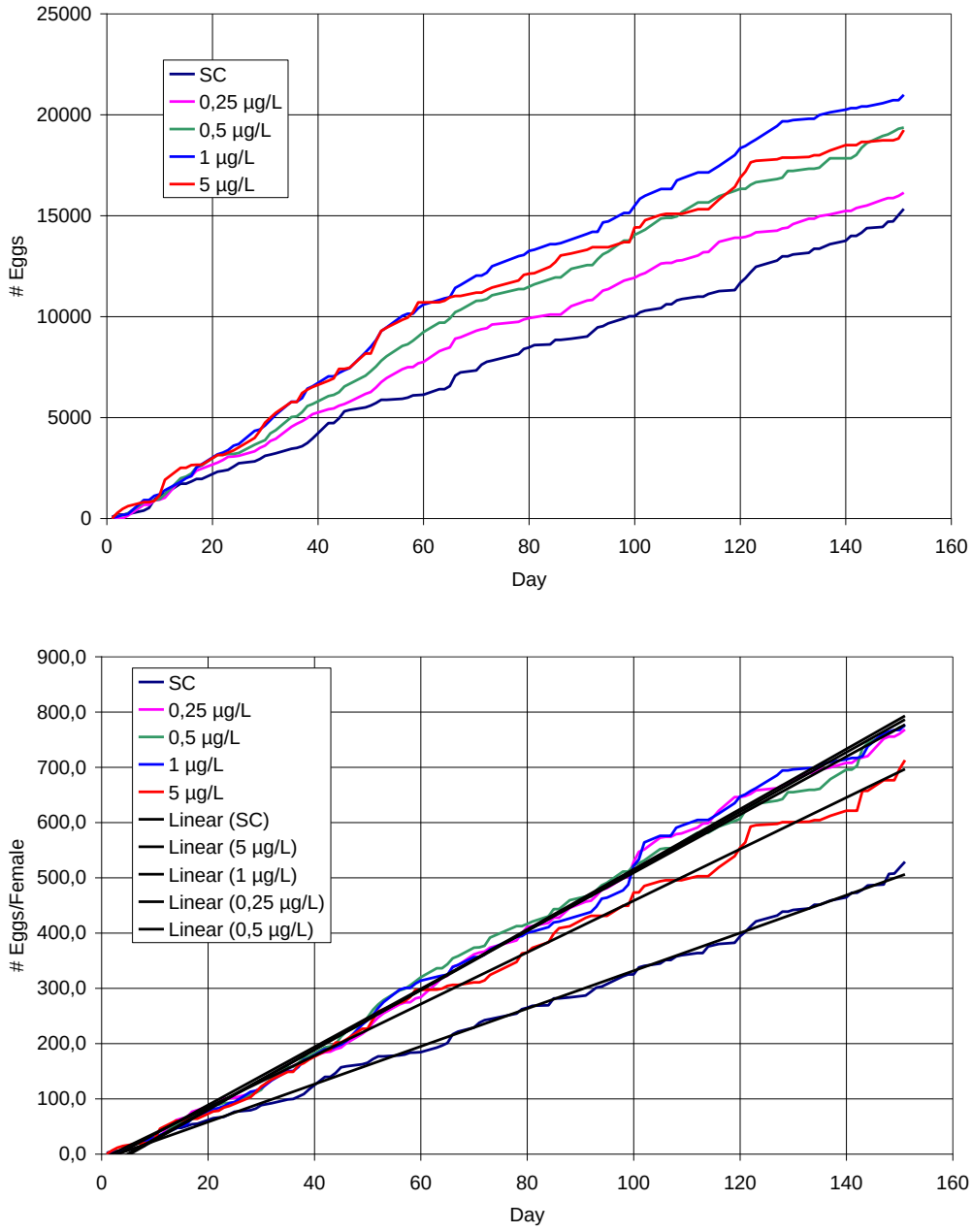

Figure 2 : Cumulative number of eggs (CNE; top) and cumulative number of Eggs/female (CNEF) together with trend lines (bottom); mean of two replicates, each.

Table 1: Comparison of the coefficient of determination, R^2, as obtained for the CNE and CNEF trendlines.

	R^2	
	CNE #Eggs	CNEF # Eggs/female
SC	0,9966	0,9977
0,25 µg/L	0,9877	0,9963
0,5 µg/L	0,9902	0,9965
1 µg/L	0,9867	0,9952
5 µg/L	0,9779	0,9962

The analysis described so far was done for both the egg numbers and clutch numbers, as well as for the two replicates and the mean of the two replicates.

Another part of the statistical analysis dealt with question about how to assess the ECx when used for promoting effects. Theoretically, promoting effects can range from slightly greater than 0% up to several hundred percent – the upper limit is not known. Therefore, it appeared reasonable to take some upper limit from the snails themselves: the reproduction from snails in the main spawning period. This high reproduction intensity was used to assess how much of the maximum reproduction was shown by snails at 20° and under presence of BPA in the period outside of the main spawning period. Since no data on the reproduction during the main spawning period at 20°C existed, results from an experiment at 22°C were used to estimate the reproduction in the main spawning period at 20°C. For preliminary estimations this appeared reasonable, since the experiments at 22°C differed from the 20°C experiments only with respect to temperature; all other environmental factors were kept at the same level (Oehlmann et al. 2000). In so doing, the reproduction rates at 22°C were calculated for both the main spawning period (Figure 3, red curve, fixed by Oehlmann in the original data) and the periods outside of the main spawning period (Figure 3, purple curves). The reproduction rate is shown by the slope parameter in respective equations in the figure.

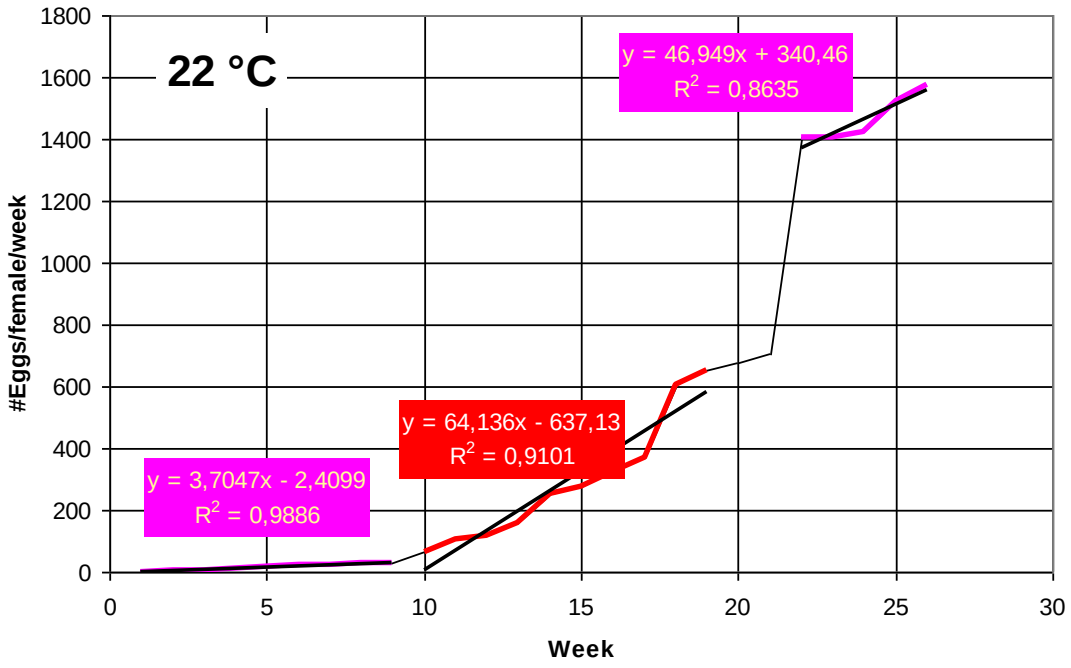

Figure 3 : Cumulative number of eggs per female (CNEF) as obtained for 22 °C. The quite different shape of the curve reative to those at 20°C is due to the removal of snails for investigation. Red curve: main spawning period; purple curves: spawning curves outside of the main spawning period.

Because one cannot be sure, that the reproduction rate after week 21 returned already to the normal rate outside the main spawning period, a factor was calculated only between the rates in the main spawning period and that in the period before the main spawning period. It turned out that the reproduction rate increased 17,3 fold in the main spawning period. This factor was used to estimate the main-spawning reproduction rate at 20°C.

Computation of Effect Values (ECx)

The computation of EC-values (e. g. the EC10 and EC 50) could not be done by standard methods. There was no visible concentration/response relationship in the BPA treatments; only 4 concentrations with nearly equal response. Although functions could have easily been found that fit the data quite well (e.g. the Weibull function; Oehlmann et al. 2006), no information is available about the true course of the response between 0 and 0,25 µg/L BPA. Many other functions might also be found, each of which leading to different hypotheses about the course of the response and possible effect values. The simplest assumption, leading to a rough estimate of EC values, is the linear interpolation between the solvent control and the first significantly different treatment (Figure 4).

The EC10 was calculated from the equation shown in Figure 4 with y = (mean of control)*1.1 (= solvent control + 10% increase) and solving for x as the concentration causing the 10% increase (=EC10).

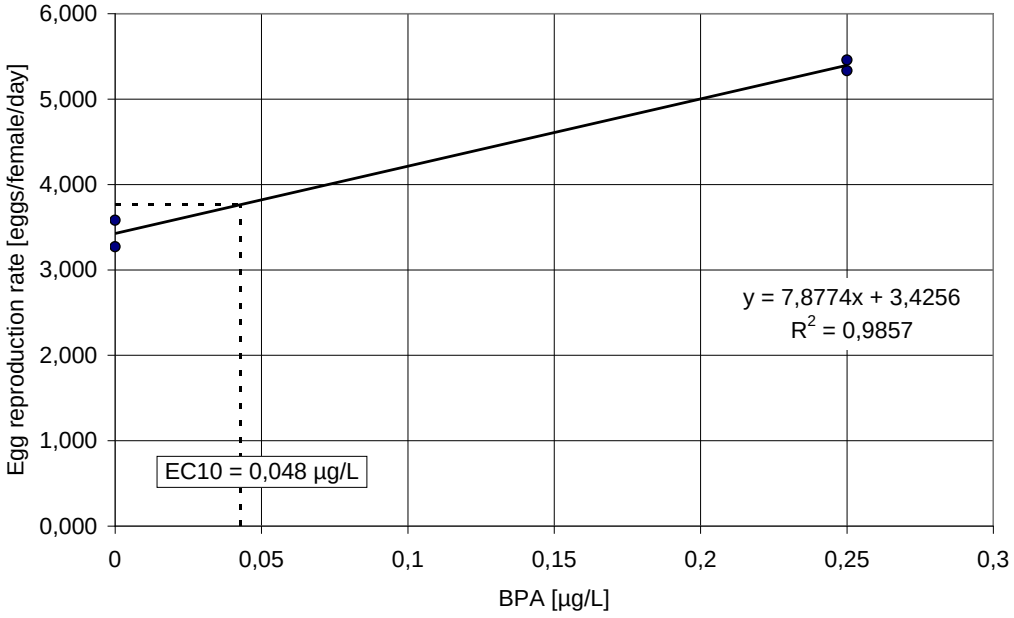

Figure 4 : Linear relationship between the solvent control (0 µg/L) and the 0,25 µg/L BPA treatment; the EC10 determination is exemplified graphically; to compute EC-values the shown equation was used

Computation of Threshold Values (NOEC)

In order to determine threshold values (NOEC) the distribution of data and variance homogeneity had to be checked, which was performed by the Shapiro Wilk's test and Bartlett's test procedure. The prerequisites normality distribution of data and variance homogeneity proved to be fulfilled in all cases, the NOEC estimations were computed using parametric tests. It turned out, that the test of choice, the William's Test procedure, which uses maximum likelihood estimated of the treatment means failed to find a decreasing or increasing order of means - in line with the above consideration on the lacking concentration/response relationship. Therefore, as an additional test the Bonferroni-t test after Holm (1979) was conducted.

The calculations were done using MS-EXCEL and ToxRat Professional 2.10 (ToxRat Solutions GmbH, 2009).

Mortality, Sex Ratio and Superfemales

Survival Curves

The day of mortality was recorded by the authors for each snail found dead in a container. From this data survival curves of snails could be established (Figure 1; example in Annex Table 1), which were used to normalize the clutches and egg production to the females alive, as was described above.

Computation of Effect Values (ECx)

For the total mortality, the proportion of females and the number of superfemales a concentration-response analysis was preformed using the classical probit method (together with Abbott's correction of control mortality. In addition, for the mortality (survival) analyses were performed at different time points.

Generally, no clear concentration/response relationship was observed in a sense that with increasing BPA concentration an increasing mortality was to be observed. As consequence, probit analyses failed to find appropriate fits in all cases (slope of the probit curve not significantly different from zero; $p > 0,05$; negative slopes, too high variability; for a summary see Annex Table 3).
The calculations were done using ToxRat Professional 2.10 (ToxRat Solutions GmbH, 2009).

Computation of Threshold Values (NOEC)

The LOEC/NOEC calculations were done using a Williams test with the arcsine-transformed survival rate in each replicate. This test uses the information of variance between replicates and proved to be more powerful than the Bonferroni-χ^2 Test. The prerequisites for the arcsine-transformation of survival (sample sizes ≥ 30) were fulfilled for this test (Horn & Volland 1995). The calculations were done using ToxRat Professional 2.10 (ToxRat Solutions GmbH, 2009).

Arcsine-transforms of mortality and superfemale rate as well as the reproduction rate were used to test possible partial correlations between endpoints using SPSS Statistics 17.0 (SPSS Inc.)

Analytical determination of Bisphenol A

With half-lives of 2,5 to 4,2 hours the BPA concentration decreased rapidly in the experimental containers showing a nearly first-order kinetics (Table 2; Figure 5).

Table 2: Analytical results for BPA after at a day one month after start as provided by the authors (SC: solvent control).

	BPA [ng/L]				
Time (h)	SC	250	500	1000	5000
-0,25	0	0	0	0	80
0,25	0	270	510	1100	4900
1	30	240	470	990	4400
2	0	190	410	820	3800
4	0	110	260	540	2400
8	0	40	110	230	1200
12	0	1	30	40	480
24	0	1	1	1	110

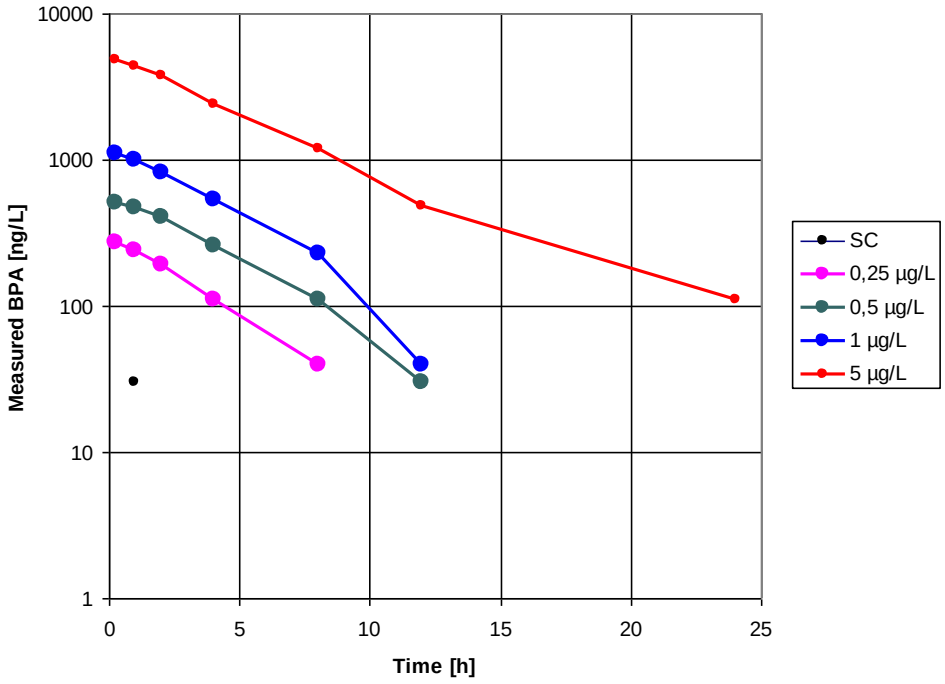

Figure 5 : Dissipation curves at 20 °C as derived from the results in Table 2; the linear characteristic of the ln of the BPA concentration on the time indicates points to a first-order dissipation kinetics.

This behaviour of BPA raises the question about the true threshold BPA concentration for the observed effects. An estimation of this threshold is difficult since no information about the uptake of BPA and internal concentration evoking the BPA effects is available. Although not experimentally proven, for the present report it is assumed that the time-weighted average (TWA) BPA-concentration is a better estimate of the exposure concentration than the nominal one. Hence, in case threshold and effect concentrations are reported, the TWA concentration is given in brackets after the nominal one.
The TWA was determined after Eq. 4: the BPA concentration of the time period i, C_i, is multiplied by the length of period i, t_i [h], and summed up. The sum is then divided by the duration of the entire measurement period ,$\sum t_i$.

Eq. 4

$$TWA = \sum(C_i * t_i) / \sum t_i$$

Table 3 presents TWAs for the considered nominal BPA concentrations. It turns out that on average the TWA is about 13% of the nominal concentration. Figure 6 gives a graphic representation of Table 3 and an equation to calculate the TWA from the nominal BPA concentration.

Table 3: Nominal vs. time-weighted average (TWA) concentrations of BPA as derived from the results in Table 2 (SC: solvent control; t/2 BPA half-live).

Nominal [µg/L]	t/2 [h]	TWA [µg/L]	TWA [ng/L]
SC		0	0,0
0,25	2,76	0,028	28,1
0,50	2,89	0,070	69,8
1.00	2,55	0,138	138,1
5.00	4,21	0,669	669,3

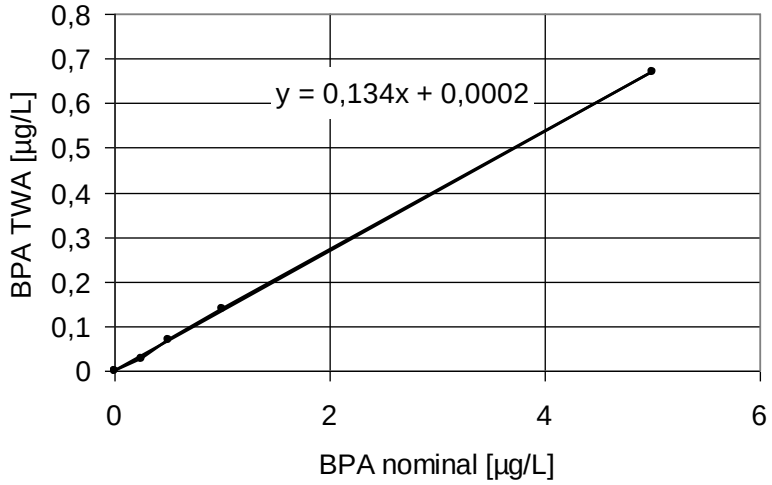

Figure 6 : Relation of the time-weighted-average (TWA) on the nominal concentration, the shown equation was used to compute TWA from nominal concentrations.

Effects of Bisphenol A on Biological Endpoints

Reproduction

Egg Reproduction Rate (ERR)

The reproduction rate determines the reproductive output over time and shall be considered first. It was already shown by Figure 2 that the cumulative number of eggs per female is a strong linear function of time in all treatments. R^2s of greater than 0,99 indicate that the reproduction rate was nearly constant over time within all experimental vessels with almost no variability (unexplained variability < 1%; Figure 7; Table 4 - except the 1 µg/L replicates). In the 1 µg/L-treatment, the reproduction rate from day 100 onwards was more variable. Due to an extremely high increase in eggs, which was to be observed during a short period around day 100, the two replicates diverged more than others.

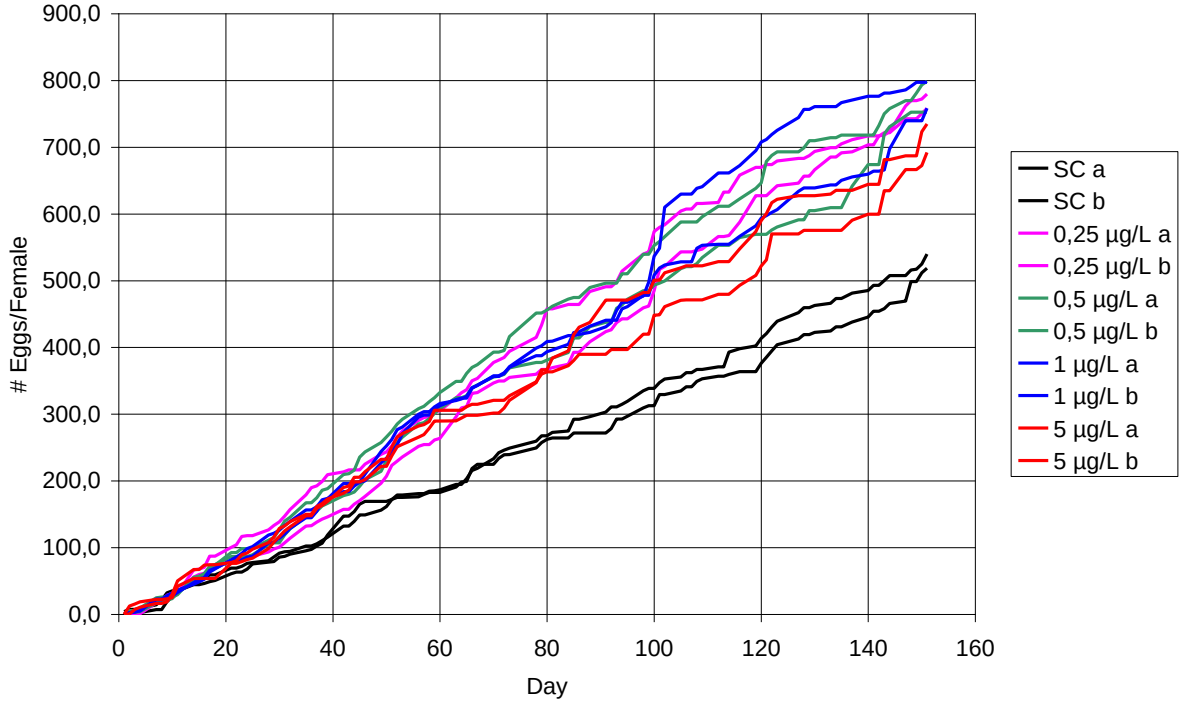

Figure 7 : Cumulative number of eggs per female (CNEF); a, b: replicate a and b of a treatment, each.

Table 4: Comparison of the coefficient of determination, R^2, the coefficient of regression, b, and it's standard error, s(b) as obtained for the CNEF trendlines. The coefficient b corresponds to the slope of the line and is the ERR [egg/female/day]

BPA [µg/L]	R^2	b	s(b)
SC	0,995	3,270	0,021
	0,992	3,581	0,017
0,25	0,994	5,333	0,039
	0,993	5,457	0,045
0,50	0,995	5,525	0,039
	0,994	5,009	0,038
1,00	0,995	5,051	0,035
	0,988	5,886	0,065
5,00	0,995	4,916	0,036
	0,992	4,449	0,039

The ERR was higher in the BPA treatments, but showed no clear relation on the concentration (Table 5; Figure 8). Already in the lower three treatments the reproduction rate increased from 3,9 to a level of 5,4 eggs/female/day on average, whereas in the highest treatment a decline in the reproduction rate down to 4,7 eggs/female/day was to be observed.

Table 5: %Increase of reproduction rate as found in the solvent control (SC) and the BPA treatments

Treatm.[µg/L]	Mean	Std. Dev.	n	%Increase
SC	3,425	0,2199	2	
0,25	5,395	0,0880	2	57,5
0,50	5,267	0,3650	2	53,8
1,00	5,468	0,5908	2	59,6
5,00	4,682	0,3305	2	36,7

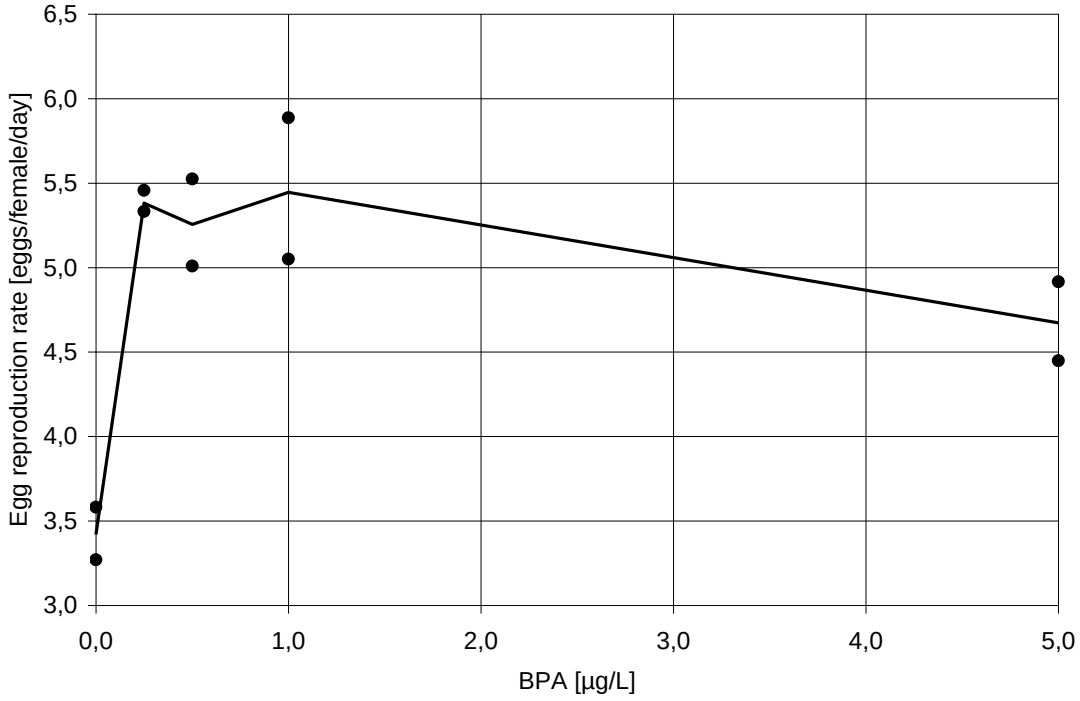

Figure 8 : ERR as found in the solvent control (0 µg/L) and the BPA treatments; dots: replicate values; line: mean per treatment.

No-Observed-Effect Concentration

The replicates corresponded to the normal distribution (Shapiro Wilk's test; p = 0,738) and their variances were homogeneous (Bartlett's test; p = 0,716). Therefore the Williams test was applied to determine the NOEC. This test uses the trend in the response and according to this generates a monotonous increasing or (in case of inhibitions) decreasing order of means – the so-called max. likelihood estimations of the "true" means (LhM; Table 6). In the present data, it found a monotonous concentration/response relationship, but neither increasing nor decreasing. A NOEC was not to fix and the threshold was found to be lower than 0,25 µg/L (0,028 µg/L). Minimum detectable differences between 26,5 and 29,1 % point to the effect size that was detectable/"recognized" by this statistical test.

Since the Williams test failed to create a concentration/response relation, the Bonferroni-t test after Holm (1979) was performed in addition. This test came to analogous results as the

Williams test, confirming that the Williams results are not only due to the max. likelihood estimation of the means (Table 7).

Table 6: Comparison of treatments with "SC" by the t test procedure after Williams. Significance was Alpha = 0,05, one-sided greater; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to SC (in percent of SC); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case t > t* (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
SC	3,859	0,50873						
0,25	5,395	0,50873	5	5,203	26,562	2,64	2,02	+
0,50	5,267	0,50873	5	5,203	28,236	2,64	2,14	+
1,00	5,468	0,50873	5	5,203	28,816	2,64	2,19	+
5,00	4,682	0,50873	5	5,203	29,119	2,64	2,21	+

+: significant; -: non-significant

Table 7: Multiple sequentially rejective comparisons of treatments with "SC" by the t test procedure. Significance was Alpha = 0,05, one-sided greater; Mean: arithmetic mean; n: sample size; s²: variance; %MDD: minimum detectable difference to SC (in percent of SC); t: sample t; p(t): probability of sample t for Ho: μ1 = μ2; Alpha(i): adjusted significance levels; the differences are significant in case p(i) <= Alpha(i) (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean	s²	df	%MDD	t	p(t)	Alpha(i)	Sign.
SC	3,425	0,130						
0,25	5,395	0,130	5	30,725	5,47	0,001	0,017	+
0,50	5,267	0,130	5	27,013	5,12	0,002	0,025	+
1,00	5,468	0,130	5	33,418	5,68	0,001	0,013	+
5,00	4,682	0,130	5	21,183	3,49	0,009	0,050	+

Effect Concentrations

The relative increase in egg reproduction rate between 0,25 and 1 µg/L was between 54 and 60%, whereas it was only 37% in the highest concentration (Table 8). In order to assess these observed increases, it appeared reasonable to relate them to the maximum egg reproduction rate in the main spawning period (MSP) of the snails. Because no data about the reproduction in the MSP at 20°C were generated, the egg reproduction rate in the solvent control was estimated from an experiment at 22 °C (see Figure 3). At 22 °C the egg reproduction rate increased 17,3-fold from the normal rate to the rate in the MSP. The same factor was used to estimate the reproduction rate in the solvent control at 20 °C during the MSP (SC MSP20; 3,42 * 17,3 = 59,2). Between 0,25 and 1 µg/L the egg reproduction rate was around 9% of the estimated SC MSP20 (around 8% at 5 µg/L). In the solvent control at 20 °C the reproduction rate was about 6% of the SC MSP20. It appears that not the full reproductive potential of the snails was evoked in the BPA treatments.

Table 8: Comparison of the egg reproduction rates relative to the solvent control (SC) and the estimated SC-egg reproduction rate in the mean spawning period at 20°C (SC MSP20; estimation method see text).

20 °C	Reproduction rate [eggs/female/day]	% Increase relative to SC	% SC MSP 20
SC	3,42	-	5,8
SC MSP20	59,2	-	-
0,25 µg/L	5,38	57,4	9,1
0,50 µg/L	5,26	53,7	8,9
1,00 µg/L	5,44	59,2	9,2
5,00 µg/L	4,67	36,6	7,9

Estimates of the EC10, EC20 and EC50, obtained by linear interpolation between the solvent control and the first significantly different treatment (0,25 µg/L; Figure 10) are given in Table 9 both for thr nominal and the TWA concentrations. The effect sizes of 10, 20 and 50% can be assessed by a comparison of the calculated egg reproduction rate (calculated at the respective EC) with the SC MSP20. It turns out that the EC10, EC20 and EC50 correspond to effects, the size of which is 6,4; 6,9 and 8,7 % of the estimated rate at the SC MSP20.

Table 9: Comparison of the calculated egg reproduction rates (calc. ERR) at the EC10, EC20 and EC50 relative to the solvent control (SC) and the estimated SC-egg reproduction rate in the mean spawning period at 20°C (SC MSP20).

		SC	3,42		
		SC MSP20	59,17	% Increase	% of estim.
µg/L	Nominal	TWA	calc. ERR	rel. to SC	MSP
EC10	0,043	0,0059	3,76	10,0	6,4
EC20	0,086	0,0117	4,10	20,0	6,9
EC50	0,216	0,0292	5,13	50,0	8,7

Cumulative Number of Eggs per Female (CNEF)

No-Observed-Effect Concentration

A closer analysis of the CNE was not performed, because it is biased by an unequal number of females. The reproductive output over time, measured as cumulative number of eggs per female (CNEF), depends on the reproduction rate and the time. Therefore the concentration response/relationship changed over time (Figure 9). Before day 50 no signs of an increase in the CNEF were visible and the NOEC was > 5 µg/L (Table 10). Thereafter, all of the BPA treatments showed a significantly increased reproduction (NOEC < 0,25 µg/L). The size of the effect steadily increased over time and was higher in between 0,25 and 1 µg/L . The increase in effect size can easily be demonstrated by the t-value obtained from the Williams test. The t-value is the standardised difference between a treatment and the solvent control. The t-value markedly increased over time (Table 10, Figure 10), suggesting that effects became stronger.

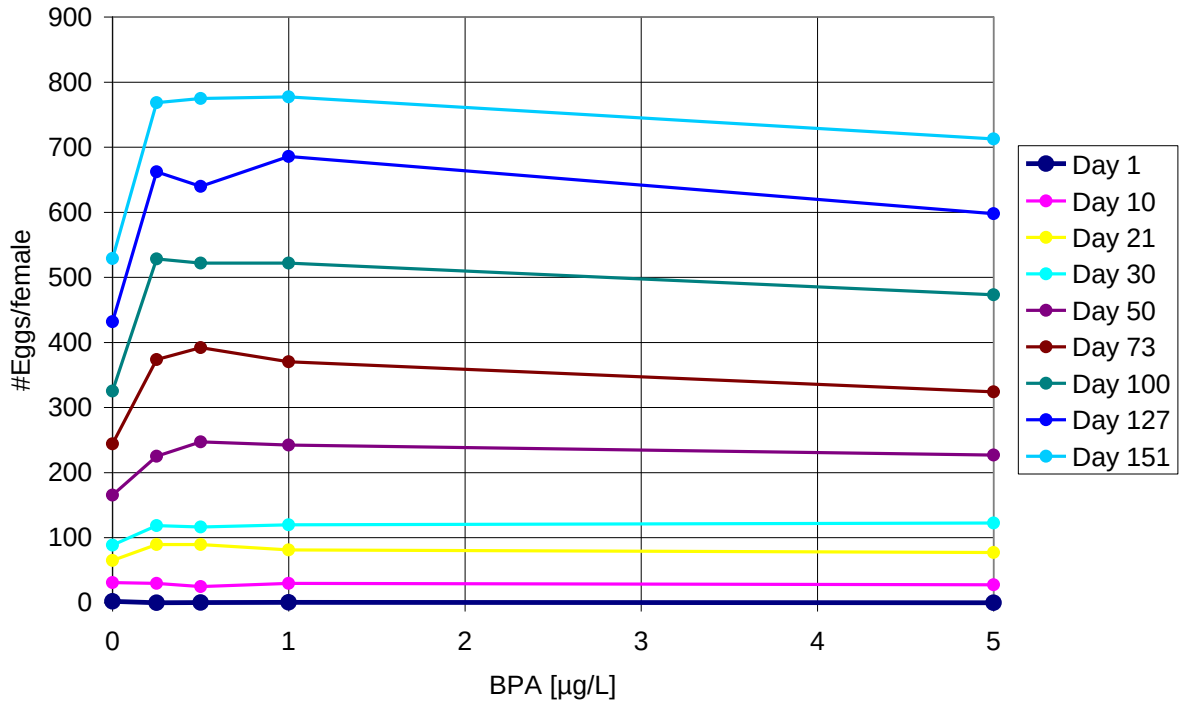

Figure 9 : Cumulative number of eggs as dependent on the BPA concentration and as found at different time point during the experimental period.

Table 10: Results of the Shapiro Wilk's and Bartlett test and comparison of the standardised difference between the solvent control an the BPA treatments, expressed as the t-value from the Williams test, as found at different time points during the experimental period. In addition the NOEC is given in terms of the nominal and time-weighted average concentration.

			BPA [µg/L]									
	Shapiro	Bart-	0,25		0,5		1		5		NOEC	
Day	Wilk's p	lett's p	t	sig.	t	sig.	t	sig.	t	sig.	nominal	TWA
1-10	0,577	0,866	-0,34	-	-1,55	-	-0,29	-	-0,79	-	> 5,0	>0,669
1-21	0,691	0,171	3,28	+	3,26	+	2,16	-	1,60	-	> 5,0	>0,669
1-30	0,815	0,648	2,11	-	1,93	-	2,15	-	2,31	-	> 5,0	>0,669
1-50	0,591	0,680	3,39	+	4,68	+	4,41	+	3,50	+	< 0,25	<0,028
1-73	0,601	0,139	6,65	+	7,57	+	6,45	+	4,07	+	< 0,25	<0,028
1-100	0,640	0,845	5,15	+	4,99	+	4,97	+	3,76	+	< 0,25	<0,028
1-127	0,536	0,844	4,33	+	3,91	+	4,78	+	3,10	+	< 0,25	<0,028
1-151	0,273	0,949	9,48	+	9,75	+	9,83	+	7,29	+	< 0,25	<0,028

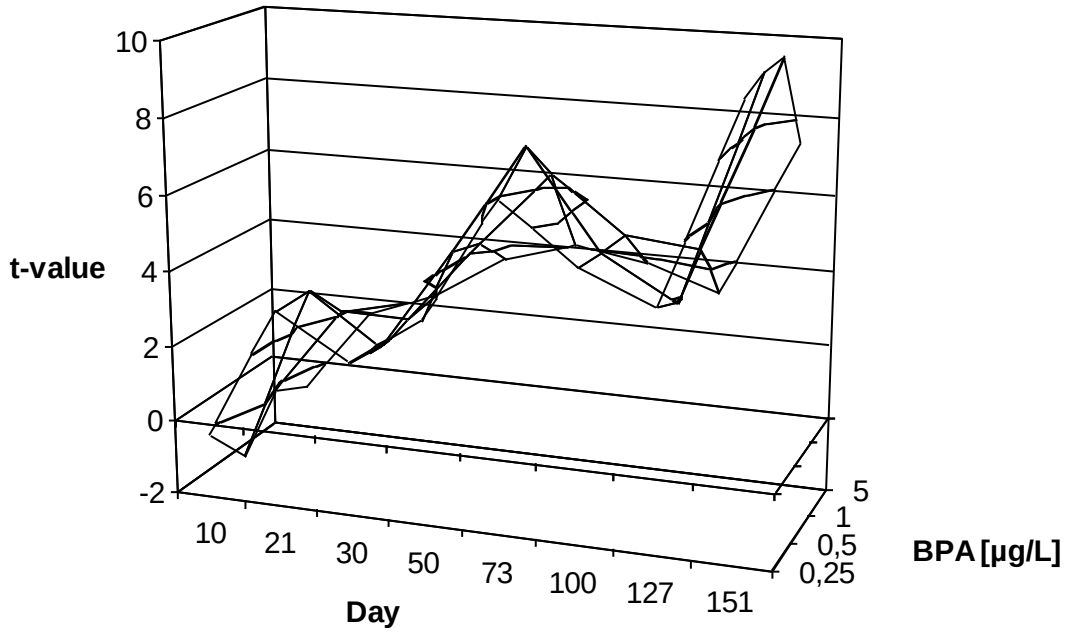

Figure 10 : Development of the standardised difference between the solvent control an the BPA treatments, expressed as the t-value from the Williams test, over time.

Effect Concentrations

As already the NOEC also the effect concentrations for the CNEF proved to be dependent on the time. Only time points between day 50 and 151 are considered since here the first treatment was significantly different from the solvent control (Table 11,Figure 11). The EC10s ranged between 0,038 and 0,068 µg/L (5,5 and 9,4 ng/L), the EC20s between 0,079 and 0,137 µg/L (10,8 and 18,6 ng/L); and the EC50 between 0,199 and 0,345 µg/L (26,8 and 46,4 ng/L). The lowest EC-values of each effect size at day 100 did closely agree with that of the EER.

Table 11: Effective nominal and TWA concentrations as observed at various time points during the experiment

Day	EC10 [µg/L]		EC20 [µg/L]		EC50 [µg/L]	
	Nominal	TWA	Nominal	TWA	Nominal	TWA
1-50	0,068	0,0094	0,137	0,0186	0,345	0,0464
1-73	0,046	0,0064	0,093	0,0127	0,234	0,0315
1-100	0,039	0,0055	0,079	0,0108	0,199	0,0268
1-127	0,046	0,0063	0,092	0,0125	0,231	0,0312
1-151	0,055	0,0075	0,110	0,0149	0,276	0,0371

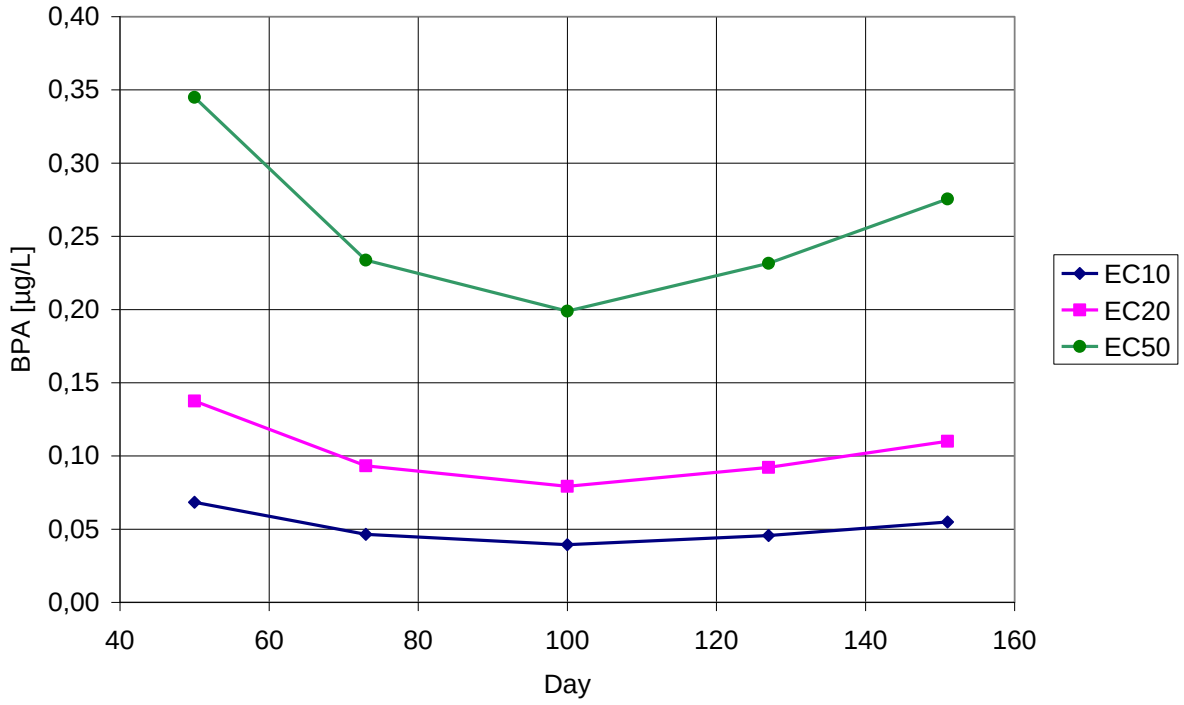

Figure 11 : Development of the effective concentrations over time.

Clutch Reproduction Rate (CRR)

The clutch production over time had the same characteristic than the egg production. The cumulative number of clutches per female was a strong linear function of time in all treatments, too. R^2s of greater than 0,99 indicate that the reproduction rate was nearly constant over time within all experimental vessels with almost no variability (unexplained variability < 1%; Figure 12; Table 12 - except one 1 µg/L replicate). In the 1 µg/L-replicate, the reproduction rate from day 100 onwards was more variable. Due to an extremely high increase in clutches, which was to be observed during a short period around day 100, the two 1 µg/L replicates diverged more than others.

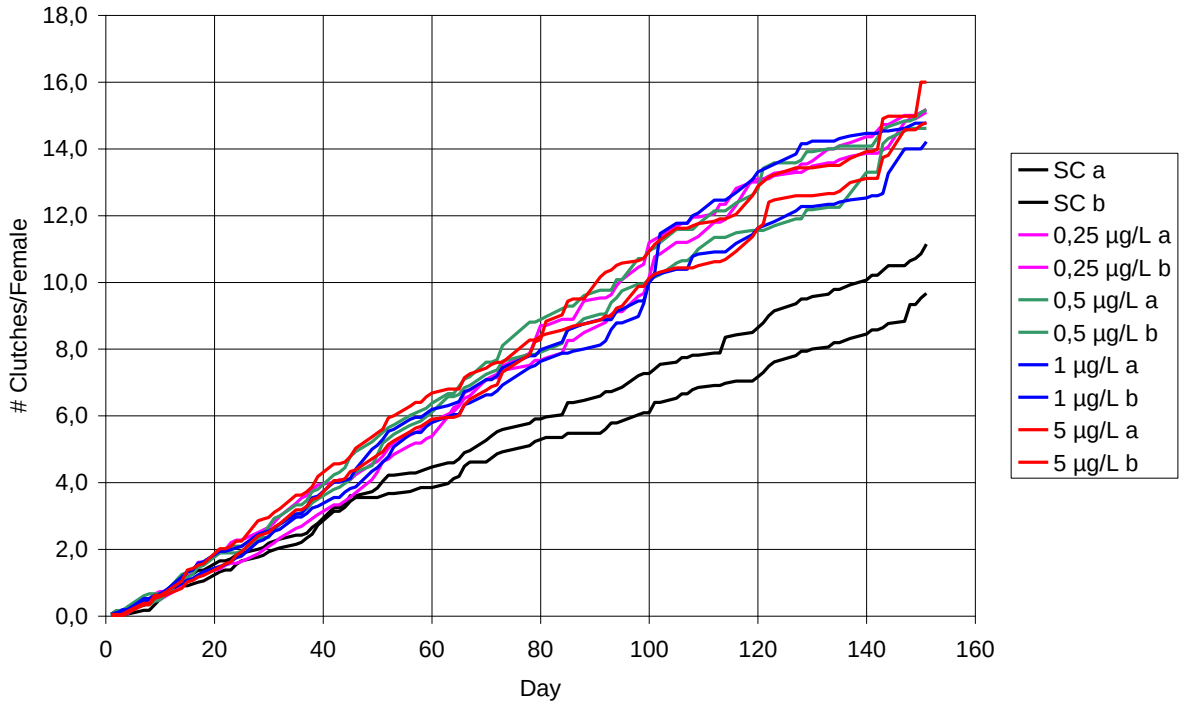

Figure 12 : Cumulative number of clutches per female (CNCF); a, b: replicate a and b of a treatment, each.

Table 12: Comparison of the coefficient of determination, R^2, the coefficient of regression, b, and it's standard error, s(b) as obtained for the CNCF trendlines. The coefficient b corresponds to the slope of the line and is the clutch reproduction rate [clutches/female/day]

BPA [µg/L]	R^2	b	s(b)
SC	0,994	0,0605	0,0005
	0,998	0,0727	0,0003
0,25	0,994	0,1099	0,0008
	0,993	0,1063	0,0009
0,50	0,994	0,1067	0,0008
	0,994	0,0990	0,0008
1,00	0,993	0,0951	0,0008
	0,987	0,1095	0,0012
5,00	0,993	0,1091	0,0009
	0,992	0,0964	0,0008

The CRR was higher in the BPA treatments, but showed no clear relation on the concentration (Table 13; Figure 13). Already in the lowest treatment the reproduction rate increased from 0,067 to a level of 10,8 clutches/female/day, whereas in the higher treatments a slight decline in the CRR was to be observed.

Table 13: %Increase of reproduction rate as found in the solvent control (SC) and the BPA treatments

Treatm.[µg/L]	Mean	Std. Dev.	n	%Increase
SC	0,067	0,0087	2	0,0
0,25	0,108	0,0026	2	62,3
0,50	0,103	0,0054	2	54,4
1,00	0,102	0,0102	2	53,5
5,00	0,103	0,0089	2	54,2

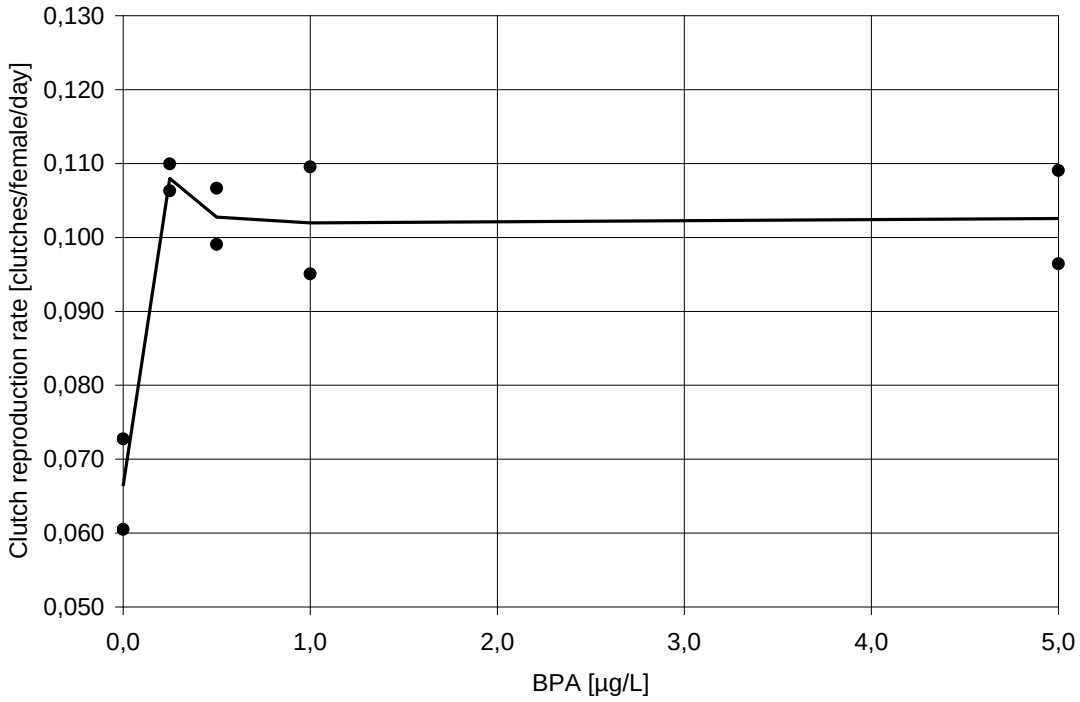

Figure 13 : CRR as found in the solvent control (0 µg/L) and the BPA treatments; dots: replicate values; line: mean per treatment.

No-Observed-Effect Concentration

The replicates corresponded to the normal distribution (Shapiro Wilk's test; p = 0,369) and their variances were homogeneous (Bartlett's test; p = 0,867). Therefore, the Williams test was applied to determine the NOEC. As with the ERR, the Williams Test found a monotonous concentration/response relationship, but neither an increasing nor a decreasing one (Table 14). A NOEC was not to fix and the threshold was found to be lower than 0,25 µg/L (0,028 µg/L).

Since the Williams test failed to create a concentration/response relation, the Bonferroni-t test after Holm (1979) was performed in addition. This test came to analogous results as the Williams test, confirming that also here the Williams results are not only due to the max. likelihood estimation of the means (Table 15).

Table 14: Comparison of treatments with "SC" by the t test procedure after Williams. Significance was Alpha = 0,05, one-sided greater; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to SC (in percent of SC); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case t > t* (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
SC	0,067	0,00768						
0,25	0,108	0,00768	5	0,104	23,235	4,87	2,02	+
0,50	0,103	0,00768	5	0,104	24,699	4,87	2,14	+
1,00	0,102	0,00768	5	0,104	25,206	4,87	2,19	+
5,00	0,103	0,00768	5	0,104	25,472	4,87	2,21	+

Table 15: Multiple sequentially rejective comparisons of treatments with "SC" by the t test procedure. Significance was Alpha = 0,05, one-sided greater; Mean: arithmetic mean; n: sample size; s^2: variance; %MDD: minimum detectable difference to SC (in percent of SC); t: sample t; p(t): probability of sample t for Ho: μ1 = μ2; Alpha(i): adjusted significance levels; the differences are significant in case p(i) <= Alpha(i) (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean	s^2	df	%MDD	t	p(t)	Alpha(i)	Sign.
SC	0,067	0,000						
0,25	0,108	0,000	5	36,674	5,40	0,001	0,013	+
0,50	0,103	0,000	5	33,719	4,72	0,003	0,017	+
1,00	0,102	0,000	5	23,247	4,64	0,003	0,050	+
5,00	0,103	0,000	5	29,646	4,70	0,003	0,025	+

Effect Concentrations

The relative increase in CRR between 0,25 and 1 µg/L and the solvent control was between 54 and 63%; the highest increase of 63% was found in the first concentration (Table 16). In order to relate the response to the maximum CCR in the main spawning period (MSP) of the snails and because no data on the clutch production at 22°C was available, the same extrapolation factor of 17,3 – as used for the ERR- was used to estimate the CCR in the solvent control at 20 °C during the MSP (SC MSP20; 0,066 * 17,3 = 1,148). Between 0,25 and 1 µg/L the clutch reproduction rate was around 9% of the estimated SC MSP20. In the solvent control at 20 °C the reproduction rate was about 6% of the SC MSP20.

Table 16: Comparison of the clutch reproduction rates relative to the solvent control (SC) and the estimated SC-clutch reproduction rate in the mean spawning period at 20°C.

20 °C	CRR [clutches/female/day]	% Increase relative to SC	% SC MSP 20
SC	0,066		5,8
SC MSP20	1,148		
0,25 µg/L	0,108	62,7	9,4
0,50 µg/L	0,103	54,8	9,0
1,00 µg/L	0,102	53,7	8,9
5,00 µg/L	0,103	54,6	8,9

Estimates of the EC10, EC20 and EC50, obtained by linear interpolation between the solvent control and the first significantly different treatment (0,25 µg/L; Figure 13) are given in Table 9 both for the nominal and the TWA concentrations. The effect sizes of 10, 20 and 50% can be assessed by a comparison of the calculated egg reproduction rate (calculated at the respective EC) with the SC MSP20. It turns out that the EC10, EC20 and EC50 correspond to effects, the size of which is 6,4; 6,9 and 8,7 % of the estimated rate at the SC MSP20.

Table 17: Comparison of the calculated clutch reproduction rates (calc. CRR) at the EC10, EC20 and EC50 relative to the solvent control (SC) and the estimated SC-clutch reproduction rate in the mean spawning period at 20°C (SC MSP20).

		SC	0,066		
		SC MSP20	1,148	% Increase	% of estim.
µg/L	Nominal	TWA	calc. CRR	rel. to SC	MSP
EC10	0,038	0,0053	0,073	10,0	6,4
EC20	0,078	0,0107	0,080	20,0	6,9
EC50	0,198	0,0268	0,100	50,0	8,7

Cumulative Number of Clutches per Female (CNCF)

No-Observed-Effect Concentration

As with the CNE a closer analysis of the CNC was not performed, because it is biased by an unequal number of females. The reproductive output over time, measured as cumulative number of clutches per female (CNCF), depends on the clutch reproduction rate and the time. Therefore the concentration response/relationship changed over time (Figure 14). Before day 30 no signs of an increase in the CNCF were visible and the NOEC was > 5 µg/L (>0,669 µg/L;

Table 18). At day 30, a NOEC of 1 µg/L (0,14 µg/L) could be determined. At day 73 the NOEC was again not to be determined (> 5 µg/L (>0,669 µg/L)). Thereafter, all of the BPA treatments showed a significantly increased reproduction (NOEC < 0,25 µg/L (<0,028 µg/L)). The size of the effect steadily increased over time and was higher in between 0,25 and 1 µg/L . The increase in effect size is again demonstrated by the t-value obtained from the Williams test. The t-value markedly increased over time (

Table 18,Figure 15), suggesting that effects became stronger.

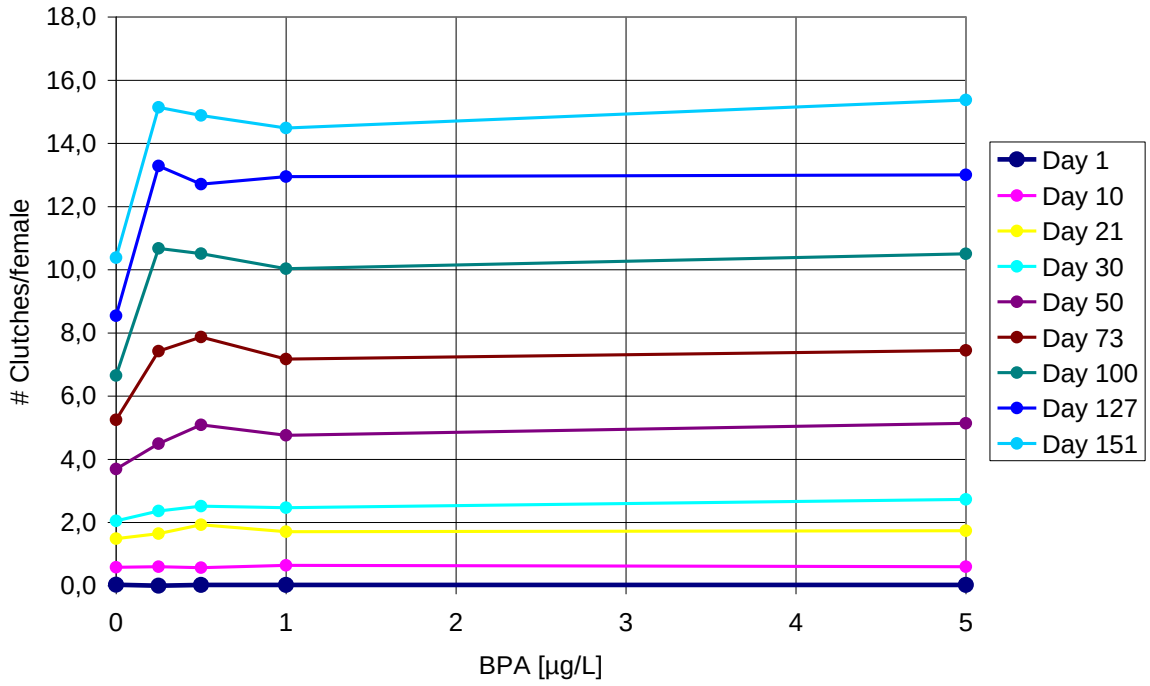

Figure 14 : Cumulative number of clutches as dependent on the BPA concentration and as found at different time points during the experimental period.

Table 18: Results of the Shapiro Wilk's and Bartlett test and comparison of the standardised difference between the solvent control an the BPA treatments, expressed as the t-value from the Williams test, as found at different time points during the experimental period. In addition the NOEC is given in terms of the nominal and time-weighted average concentration.

			BPA [µg/L]									
	Shapiro	Bart-lett's	0,25		0,5		1		5		NOEC [µg/L]	
Day	Wilk's p	p	t	sig.	t	sig.	t	sig.	t	sig.		TWA
1-10	0,588	0,89	0,50	-	0,50	-	0,75	-	0,75	-	> 5,0	>0,669
1-21	0,542	0,831	0,52	-	1,04	-	1,04	-	1,04	-	> 5,0	>0,669
1-30	0,522	0,903	1,21	-	1,65	-	1,65	-	2,43	+	1,00	0,14
1-50	0,703	0,873	2,45	+	4,08	+	4,08	+	12,23	+	< 0,25	<0,028
1-73	0,958	0,742	6,22	+	6,22	+	6,45	+	-0,26	-	> 5,0	>0,669
1-100	0,352	0,616	5,80	+	5,80	+	5,80	+	5,93	+	< 0,25	<0,028
1-127	0,525	0,475	4,65	+	4,65	+	4,65	+	4,65	+	< 0,25	<0,028
1-151	0,623	0,505	6,92	+	6,92	+	6,92	+	6,92	+	< 0,25	<0,028

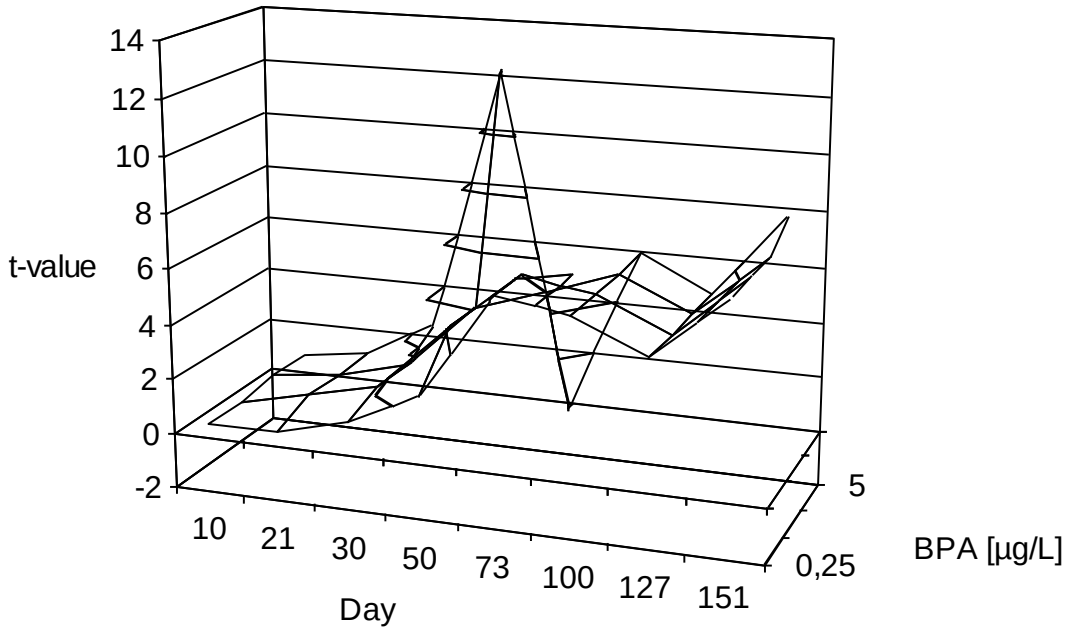

Figure 15 : Development of the standardised difference between the solvent control an the BPA treatments, expressed as the t-value from the Williams test, over time.

Effect Concentrations

As already shown for the CNEF, also in the CNCF the ECx proved to be dependent on the time. Only time points between day 50 and 151 are considered since here the first treatment was significantly different from the solvent control (Table 19; Figure 16). The EC10s ranged between 0,04 and 0,117 µg/L (5,6 and 15,9 ng/L), the EC20s between 0,082 and 0,236 µg/L (12,0 and 31,8 ng/L); and the EC50 between 0,207 and 0,592 µg/L (27,9 and 79,5 ng/L). The lowest EC-values of each effect size at day 100 did closely agree with that of the CRR.

Table 19: Effective nominal and TWA concentrations as observed at various time points during the experiment for the cumulative clutch number per female over time.

	EC10 [µg/L]		EC20 [µg/L]		EC50 [µg/L]	
Day	Nominal	TWA	Nominal	TWA	Nominal	TWA
1-50	0,117	0,0159	0,236	0,0318	0,592	0,0795
1-73	0,059	0,0081	0,120	0,0162	0,301	0,0405
1-100	0,040	0,0056	0,082	0,0120	0,207	0,0279
1-127	0,043	0,0060	0,088	0,2822	0,223	0,0301
1-151	0,053	0,0074	0,108	0,0147	0,273	0,0367

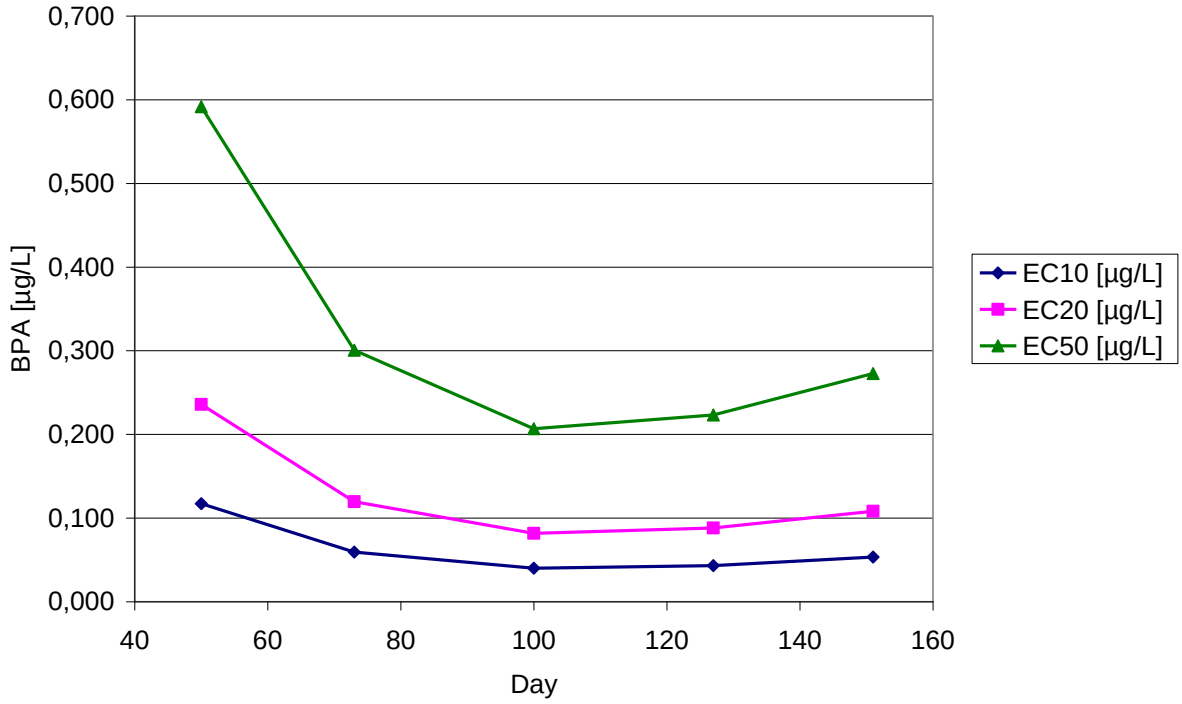

Figure 16 : Development of the effective concentrations for the cumulative clutch number per female over time.

Eggs per Clutch

The observed increase in egg production as shown above can be due to the increase of the number of clutches and/or the increase in the number of eggs per clutch. As shown in Figure 17 there was a significant response on the increasing test item concentration with maximum increase of 12,9 % at the highest concentration ($p < 0,05$; test of the correlation coefficient r).

No-Observed-Effect Concentration

The NOEC was found to be 1 µg/L (Table 21).

Effect Concentrations

Estimates of the EC10 and EC20 were derived from the trend line as shown in Figure 17 in an analogous way as described for the aforementioned variables. EC10 was 3,80 µg/L (0,509 µg/L) and the EC20 proved to be 7,55 µg/L (1,012 µg/L) (Table 22). An EC50 was not calculated, since it would be far beyond the measured concentration range.

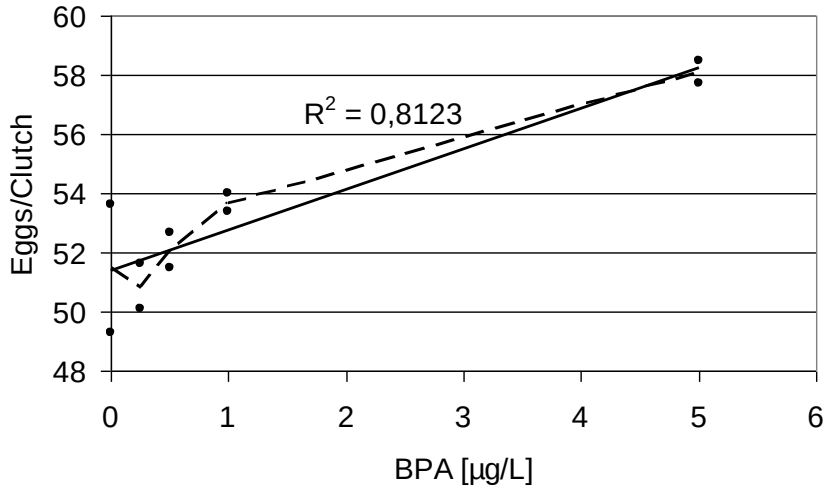

Figure 17: Number of eggs per clutch as dependent on the nominal concentration of BPA (total experimental period)

Table 20: Mean number of eggs per clutch and % increase as dependent on the BPA concentration (Std: standard deviation; n number of clutches; sc: solvent control).

BPA [µg/L]	Mean	Std	% Increase	n
SC	51,5	20,6		291
0,25	50,8	23,1	-1,2	317
0,5	52,1	21,1	1,2	372
1	53,7	22,4	4,3	391
5	58,1	23,4	12,9	415

Table 21: Comparison of treatments with "SC" by the t test procedure after Williams. Significance was Alpha = 0,05, one-sided greater; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to SC (in percent of SC); t: sample t; t*: critical t for Ho: µ1 = µ2 = ... = µk; the differences are significant in case t > t* (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	**Mean**	**s**	**df**	**LhM**	**%MDD**	**t**	**t***	**Sign.**
SC	51,461	1,53235						
0,25	50,857	1,53235	5	50,857	6,000	-0,39	2,02	-
0,50	52,077	1,53235	5	52,077	6,378	0,40	2,14	-
1,00	53,673	1,53235	5	53,673	6,509	1,44	2,19	-
5,00	58,105	1,53235	5	58,105	6,578	4,34	2,21	+

+: significant; -: non-significant

Table 22: Effective nominal and TWA concentrations as observed for the number of eggs per clutch.

EC10 [µg/L]		EC20 [µg/L]	
Nominal	TWA	Nominal	TWA
3,80	0,509	7,55	1,012

Mortality, Superfemales and Sex Ratio

The survival curves (Figure 1) of the snails can be divided in three groups: (1) the solvent control showed a nearly constant, but small mortality rate throughout the experimental period, (2) in the 0,5 µg/L treatment the snails died mainly between day 10 and day 85, whereas (3) in the remaining treatments the main mortality occurred between day 10 and day 100. There are clear hints, that the pattern two and three were due to the treatment with BPA. A reason, why the main mortality occurred in the first half of the experimental period, cannot be given. Depending of the mortality events in the solvent control and the BPA treatments the NOEC differed at the selected time point, but fell below 0,025 µg/L (i. e. was not to determine exactly; Table 23).

Table 23: Comparison of the standardised difference between the solvent control and the BPA treatments, expressed as the t-value from the Williams test with arcsine-transformed survival rates, as found at different time points during the experimental period. In addition the NOEC is given in terms of the nominal and time-weighted average concentration.

	BPA [µg/L]									
	0,25		0,5		1		5		NOEC [µg/L]	
Day	t	sig.	t	sig.	t	sig.	t	sig.	nominal	TWA
1-21	0,00	-	0,60	-	0,60	-	0,60	-	> 5,0	>0,669
1-30	0,00	-	4,17	+	4,17	+	4,17	+	0,25	0,028
1-50	0,90	-	1,64	-	1,64	-	1,64	-	> 5,0	>0,669
1-73	1,07	-	1,34	-	1,34	-	1,34	-	> 5,0	>0,669
1-100	3,53	+	3,53	+	3,53	+	3,53	+	< 0,25	<0,028
1-127	2,16	+	2,16	+	2,16	-	2,16	-	> 5,0	>0,669
1-151	3,15	+	3,38	+	3,38	+	3,38	+	< 0,25	<0,028

The mortality per replicate until day 151 ranged between 20% in the solvent control an 40% in one of 0,25 µg/L-replicates (Table 24). The resulting total mortality per treatment (Figure 18) shows a clear increase in the BPA treatments, but no clear concentration/response relationship in a sense that with increasing BPA concentration an increasing mortality was to be observed. No ECx could be determined (for a summary see Annex Table 3).

Mortality in all BPA treatments was significantly higher ($p < 0,05$) than in the solvent control so that the determination of an NOEC was impossible (Table 25) .

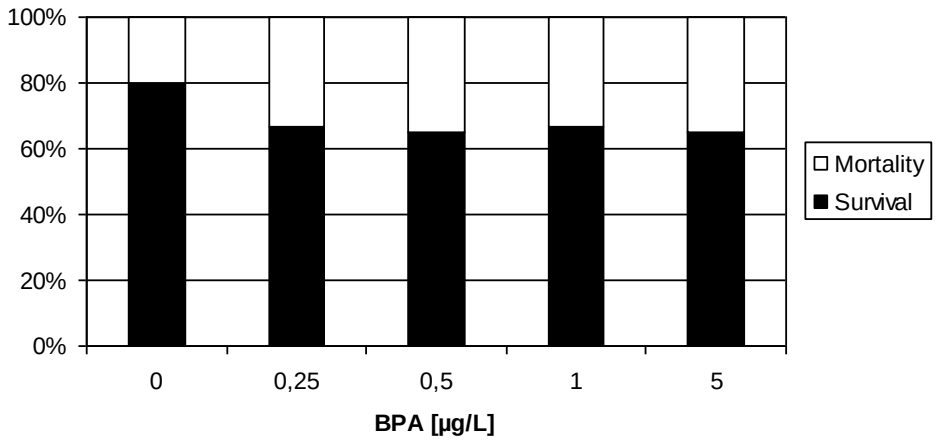

Figure 18 : Total survival and mortality during the experimental period (sum of two replicates, each); 0 µg/L: solvent control.

Table 24: %Mortality, %Superfemales and %Females as observed in replicates of the solvent control (SC) and BPA treatments

BPA [µg/L]	%Mortality	%Superfemales	% Females
SC	20,0	0,0	62,5
	20,0	0,0	58,3
0,25	40,0	0,0	61,1
	26,7	10,0	45,5
0,5	33,3	8,3	60,0
	36,7	7,7	68,4
1	33,3	14,3	70,0
	33,3	15,4	65,0
5	36,7	15,4	68,4
	33,3	7,1	70,0

Table 25: Comparison of treatments with "SC" by the t test procedure after Williams using arcsine-transformed survival. Significance was Alpha = 0,05, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to SC (in percent of SC); t: sample t; t*: critical t for Ho: µ1 = µ2 = ... = µk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	**Mean**	**s**	**df**	**LhM**	**%MDD**	**t**	**t***	**Sign.**
SC	1,107	0,04757						
0,250	0,957	0,04757	5	0,957	-8,658	-3,15	-2,02	+
0,500	0,938	0,04757	5	0,947	-9,204	-3,38	-2,14	+
1,000	0,955	0,04757	5	0,947	-9,393	-3,38	-2,19	+
5,000	0,938	0,04757	5	0,938	-9,491	-3,56	-2,21	+

+: significant; -: non-significant

Superfemales were observed at low rates only in the BPA treatments (Table 24; Figure 19). The probit analysis failed to generate valid ECx values (slope = 0; p = 0,572)

The Bonferroni-Fisher test could not determine a NOEC (> 5 µg/L; Table 26). In contrast, a NOEC of 0,25 µg/L was suggested by the Williams test (Table 27), however, the prerequisite $n \geq 30$ for the arcsine-transformation is not exactly met, since the number of investigated females and/or the replication was somewhat too low.

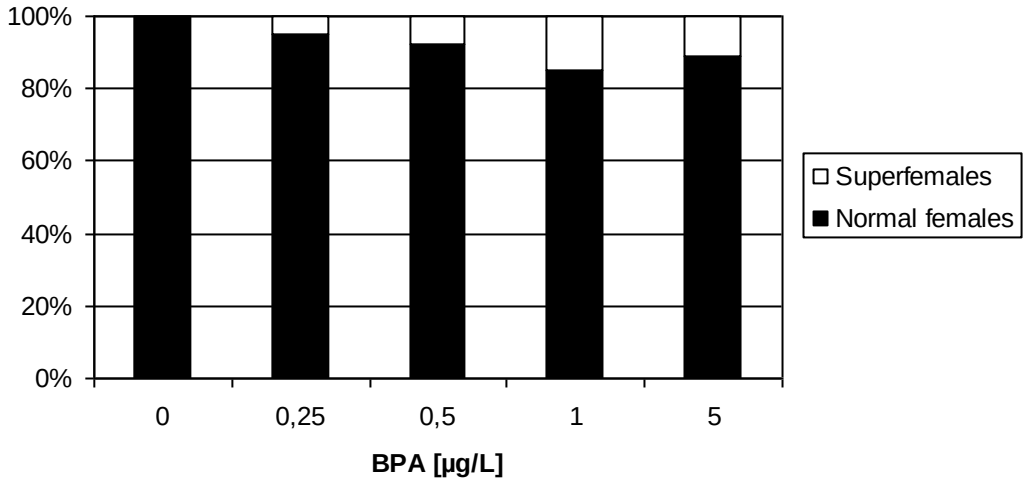

Figure 19 : Ratio of superfemales as found in the solvent control (0 µg/L) and BPA treatments (sum of two replicates, each)

Table 26: Fisher`s Exact Binomial Test with Bonferroni Correction: Pair-wise comparisons between treatment and control on the multiple significance level (alpha is 0,05; one-sided greater). Pair-wise comparisons are performed sequentially using the adjusted Alpha* (= alpha/(k-1); k: number of comparisons (after Holm 1979)); Ho (no effect)is accepted, if the probability p > Alpha*.

Treatm.[µg/L]	Dissected	Normal	Superfem	% Superfem	p	alpha*	sign.
SC	20	20	0	0,0			
0,250	21	20	1	4,8	0,512	0,050	-
0,500	25	23	2	8,0	0,303	0,025	-
1,000	27	23	4	14,8	0,098	0,013	-
5,000	27	24	3	11,1	0,180	0,017	-

+: significant; -: non-significant

Table 27: Comparison of treatments with "SC" by the t test procedure after Williams. Significance was Alpha = 0,05, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to SC (in percent of SC); t: sample t; t*: critical t for Ho: µ1 = µ2 = ... = µk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean*)	s	df	LhM	%MDD	t	t*	Sign.
SC	1,57	0,1102						
0,250	1,41	0,1102	5	1,41	-14,14	-1,46	-2,02	-
0,500	1,28	0,1102	5	1,28	-15,03	-2,60	-2,14	+
1,000	1,18	0,1102	5	1,20	-15,34	-3,32	-2,19	+
5,000	1,23	0,1102	5	1,20	-15,50	-3,32	-2,21	+

+: significant; -: non-significant

*) Arcsine-transformed ratio of normal females

Except for the 0,25 µg/L-treatment, the proportion of females was higher in all remaining treatment and the solvent control (Table 24; Figure 20). The probit analysis failed to generate valid ECx values (slope = 0; p = 0,220)

The Bonferroni-Fisher test and Williams test could not determine a NOEC (≥ 5 µg/L; Table 28; Table 29).

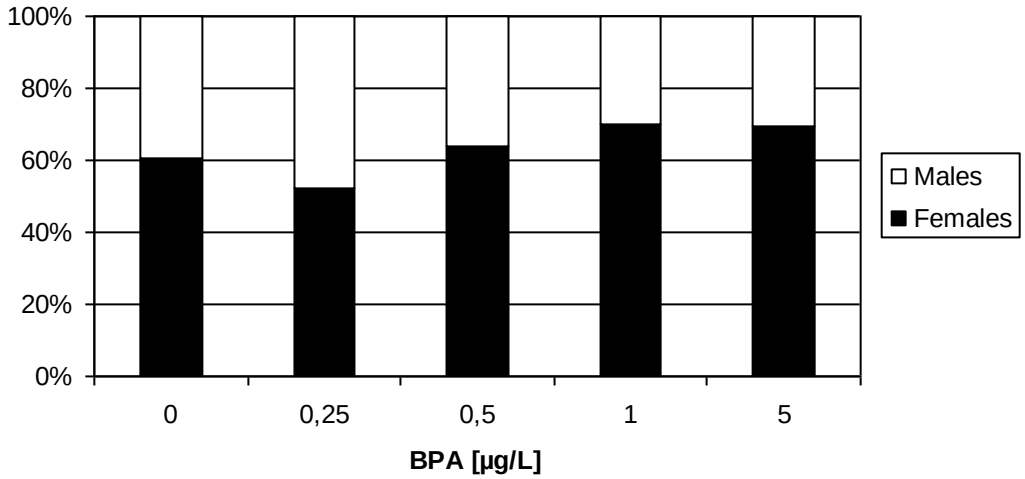

Figure 20 : Ratio of females as found in the solvent control (0 µg/L) and BPA treatments (sum of two replicates, each)

Table 28: Fisher`s Exact Binomial Test with Bonferroni Correction: Pair-wise comparisons between treatment and control on the multiple significance level (alpha is 0,05; one-sided greater). Pair-wise comparisons are performed sequentially using the adjusted Alpha* (= alpha/(k-1); k: number of comparisons (after Holm 1979)); Ho (no effect)is accepted, if the probability p > Alpha*.

Treatm.[µg/L]	Dissected	Females	Males	% Mortality	p	alpha*	sign.
SC	48	29	19	39,6			
0,25	40	21	19	47,5	0,298	0,013	-
0,50	39	25	14	35,9	0,717	0,017	-
1,00	40	27	13	32,5	0,819	0,025	-
5,00	39	27	12	30,8	0,860	0,050	-

+: significant; -: non-significant

Table 29: Comparison of treatments with "SC" by the t test procedure after Williams. Significance was Alpha = 0,05, one-sided greater; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to SC (in percent of SC); t: sample t; t*: critical t for Ho: µ1 = µ2 = ... = µk; the differences are significant in case t > t* (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean*)	s	df	LhM	%MDD	t	t*	Sign.
SC	0,89	0,0613						
0,25	0,82	0,0613	5	0,82	13,86	-1,17	2,02	-
0,50	0,93	0,0613	5	0,93	14,74	0,65	2,14	-
1,00	0,96	0,0613	5	0,96	15,04	1,21	2,19	-
5,00	0,98	0,0613	5	0,98	15,20	1,50	2,21	-

+: significant; -: non-significant

*) Arcsine-transformed proportion of females

Discussion

Major questions arising from the results include the BPA concentration, which may have caused an effect, the statistical design of the study and the analysis and finally which effects of BPA are probably direct or indirect.

BPA concentration

It was found that the BPA concentration steeply decreased, soon after the fresh medium with BPA was added to the snails. Thus, nominal concentrations are not appropriate to quantify the possible effects of BPA. Also the time-weighted average of the BPA concentration is not seen as the final solution, since it remains unknown, which internal concentration at the target sites is directly proportional to the response of the snails. This information could probably be generated by pharmacokinetic modelling, which would be highly recommendable. Preuss et al. (in prep.) showed that pharmacokinetic modelling allowed to model effects of nonylphenol on *Daphnia* rather than the use of the effect concentrations derived from the concentration/response relationship or the time-weighted average concentration.
For the time being, the author of the present report recommends to accept that the BPA concentration eliciting the effects is probably between the TWA and the nominal concentration. In addition, to meet the scientific needs of this important regulatory problem, the internal concentrations for evoking the observed effects have to be clarified urgently.

Statistical results

The statistical analysis proved to be problematic. It cannot be stated that this was only due to the statistical design chosen by Oehlman et al (2006) or the underlying fact that the response on the BPA treatment does generally not follow the classical dose/response scheme when BPA acts as an endocrine disrupter. The selected statistical design with 4 treatments and 2 replicates, each, does not meet the minimum requirements of an OECD study which normally is conducted with 5 treatments and at least 3 replicates (6 replicates in the control, to increase the statistical power of the design; e.g. the algae growth inhibition test, OECD 201). In many cases the replication is much higher (e.g. 10 replicates per treatment in the Daphnia reproduction test, OECD 211).
However, this was “compensated” by the fact that in the reproduction variables already in the lowest tested concentration a significant increase was to be observed, i.e. indicating that there was an effect, but a NOEC could not be exactly determined. A normal concentration/response study with pesticides or chemicals would not be accepted, in case an unequivocal NOEC is required.
The response of the reproduction variable in the BPA treatments was found to be significantly increased, but did not follow a concentration/response pattern and the maximum-likelihood estimation of the mean response by the Williams test led to the same response in all treatments. Therefore, because the course of the response between the solvent control and the first BPA treatment is unknown, no of the commonly used concentration/response functions were seen as appropriate. As an alternative, a linear relationship between the response in the solvent control and the first treatment was assumed and the ECx computations were based on that relation. Therefore, the ECx values are only raw estimates.

The observation that already the first treatment showed a significant response and the responses at higher treatments did not show a more intense response could be due to either an inappropriate concentration selection (no range finding test) or to the specific action of BPA. This important question remains yet unresolved and for the time being leads to a high uncertainty. Consequently, mechanistic studies should be initiated to find this out.

In addition, the ECx here stands for a promotion of the reproduction rather than an inhibition, as is the case in classical studies with pesticides and other chemicals, where it is used as a toxicity measure. The maximum possible intensity of the promotion cannot be given directly. A general recommendation, how to describe promoting effects by an appropriate parameter

is yet lacking. Nonetheless, the ECx was used here also for the promoting effects. A species-specific measure to assess the promoting effects in the present case was seen in the reproduction intensity exhibited in the main spawning period of the snails. This was not regarded as a possibility by others, e.g. Oehlmann et al. (2006) so far, and its value should be discussed by the scientific community further.

According to the discussions so far, the effect parameters given in the following summary table (Table 30) should be taken with care.

Table 30: Summary of the statistical results as found for the cumulative number of eggs per female (CNEF), the egg reproduction rate (ERR), the cumulative number of clutches per female (CNCF), the clutch reproduction rate (CRR), the number of eggs per clutch, the mortality, the superfemale induction and the sex ratio; concentration are nominal or time-weighted averages (TWA)

CNEF		NOEC [µg/L]		EC10 [µg/L]		EC20 [µg/L]		EC50 [µg/L]	
	Day	Nominal	TWA	Nominal	TWA	Nominal	TWA	Nominal	TWA
	1-10	> 5,0	>0,669	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1-21	> 5,0	>0,669	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1-30	> 5,0	>0,669	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1-50	< 0,25	<0,028	0,068	0,0094	0,137	0,0186	0,345	0,0464
	1-73	< 0,25	<0,028	0,046	0,0064	0,093	0,0127	0,234	0,0315
	1-100	< 0,25	<0,028	0,039	0,0055	0,079	0,0108	0,199	0,0268
	1-127	< 0,25	<0,028	0,046	0,0063	0,092	0,0125	0,231	0,0312
	1-151	< 0,25	<0,028	0,055	0,0075	0,110	0,0149	0,276	0,0371
ERR	1 - 151	< 0,25	<0,028	0,043	0,0059	0,086	0,0117	0,216	0,0292
CNCF	1-10	> 5,0	>0,669	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1-21	> 5,0	>0,669	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1-30	1,00	0,14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1-50	< 0,25	<0,028	0,117	0,0159	0,236	0,0318	0,592	0,0795
	1-73	> 5,0	>0,669	0,059	0,0081	0,120	0,0162	0,301	0,0405
	1-100	< 0,25	<0,028	0,040	0,0056	0,082	0,0120	0,207	0,0279
	1-127	< 0,25	<0,028	0,043	0,0060	0,088	0,2822	0,223	0,0301
	1-151	< 0,25	<0,028	0,053	0,0074	0,108	0,0147	0,273	0,0367
CRR	1 - 151	< 0,25	<0,028	0,038	0,0053	0,078	0,0107	0,198	0,0268
Eggs/Clutch	1 - 151	1,00	0,14	3,8	0,509	7,55	1,012	n.d.	n.d.
Mortality	1-21	> 5,0	>0,669	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1-30	0,25	0,028	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1-50	> 5,0	>0,669	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1-73	> 5,0	>0,669	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1-100	< 0,25	<0,028	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1-127	> 5,0	>0,669	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1-151	< 0,25	<0,028	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Superfemale	1 - 151	0,25	0,028	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SexRatio	1 - 151	> 5,0	>0,669	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Among the reproduction variables the clutch reproduction rate (CRR) with an EC10 of 0,038 (0,0053) µg/L BPA was the most sensitive variable in terms of the ECx, followed by the cumulative number of eggs per female (CNEF) with an EC10 of 0.039 (0,0055) µg/L BPA and the egg reproduction rate (ERR) with an EC10 of 0,43 (0,0059) µg/L BPA. Among the qualitative variables the mortality after 151 days (NOEC < 0,25 (0,028) µg/L BPA) and the induction of superfemales (NOEC = 0,25 (0,028) µg/L BPA) were the most sensitive ones.

It turned out egg and clutch reproduction rates were superior over the remaining reproduction variables since they are time-independent and nonetheless sensitive which is seen as ad-

vantageous for regulatory purposes. Otherwise there is no clear advice which of the toxicity parameters varying in time should be taken.

Further studies with this snail - from the beginning on - should focus on measuring the reproduction rates. This has been shown here to be advantageous and is seen as one measure to overcome the statistical shortcomings of the study considered in the present report. The measurement of the reproduction rates does not require long time periods, as could be shown by the rate calculation of selected time periods in the 22°C study and earlier studies on the considered snail evaluated by the author of the present study. The savings could be spent in studying more concentrations at higher replication. Other simplifications are seen in selecting copulas of snails (one male, one female) from the stock culture and observe single (or groups) of snail pairs per treatment. This would lead to a better control over what is happening during the experiment (sex dependent mortality, superfemale reproduction and mortality, etc.).

BPA Effects

The order to the author of the present study was to perform the statistical analysis of the data provided by the sponsor. Although the author of the present report has no expertise with respect to the biology and special behaviour of the studied snail species, some hypotheses shall be discussed briefly.
The findings give reason to the following hypotheses:

1. BPA causes the induction of superfemales (Oehlmann et al. 2006).
2. BPA exerts a toxic action on the snails resulting in a mortality increase (Oehlmann et al. 2006)..
3. BPA stimulates the reproduction (Oehlmann et al. 2006).
4. The higher mortality rate in the BPA treatments is due to a increased mortality in females, in particular superfemales (Oehlmann et al. 2006)..
5. The mortality increase is an indirect effect of the higher egg reproduction rate and exhaustion of females in the BPA treatments (Oehlmann et al. 2006).
6. The increased egg reproduction rates in the BPA treatment are an indirect effect due to an increased food supply caused by mortality (author of the present study).

Hypothesis 1 is true, since the induction of superfemales is a clear indication of an endocrine disrupting effect brought about by BPA. This effect followed a concentration/response relation, although statistically not significant (too few replicates). Hypothesis 2 cannot be clearly verified or falsified. The lacking concentration response relation however does not support this hypothesis. Hypothesis 3 can be true but is not the only explanation. Hypothesis 4 cannot exactly be investigated since the number of introduced males and females is unknown and thus also the sex-specific mortality. However, the sex-ratio observed at study end does not support this hypothesis, the proportion of females in the three highest BPA treatments was even higher than in the solvent control. In favour of Hypothesis 5 appears to be the observation that mortality was as concentration independent as the egg reproduction rate. Finally, Oehlmann et al (2006) described that the snails were fed ad libitum with TetraMin and additional lettuce leaves. A proof is yet lacking that the snails could really realize maximum feeding rates under the feeding regime at 20°C.

Generally, it appears that systematic studies on feeding and reproductive behaviour of *Marisa cornuarietis* are yet lacking. For example, studies about the main spawning period were done at 22°C, whereas those are lacking at 20°C. Other studies were performed at 27°C, in which no BPA effect were visible (May be that this was due to food limitation). With respect to the BPA effects, there is urgent need to perform pharmacokinetic studies and modelling of the internal concentrations responsible for the observed effects.

Conclusions

Summarising, the BPA concentration rapidly decreased in the experimental vessels, which does not allow basing the risk assessment on nominal concentrations. Using time-weighted average concentrations (TWA) gives also reasons for uncertainty because they might be closer to the internal effective concentrations but these remain unknown. TWA concentrations are given in brackets.
There are strong hints that the induction of superfemales (NOEC 0,25 (0,028) µg/L) and mortality was evoked by BPA. The sex ratio was not visibly affected.
Among the reproduction parameters the clutch reproduction (CRR) rate and the egg reproduction rate (ERR) proved to be the variables of choice to compute time-independent toxicity parameters (NOEC, ECx).
The statistical design of the study did not allow to determine NOECs (rare exceptions were found but their meaning was unclear; probably statistical artefacts). The EC10, EC20 and EC50 could only be computed using a raw estimation of the concentration/response between solvent control and the 0,25 µg/L BPA-treatment by linear interpolation.

Having in mind the methodological problems and the associated uncertainties, the clutch reproduction rate (CRR) with an EC10 of 0,038 (0,0053) µg/L BPA was the most sensitive variable, followed by the cumulative number of eggs per female (CNEF) with an EC10 of 0.039 (0,0055) µg/L BPA and the egg reproduction rate (ERR) with an EC10 of 0,43 (0,0059) µg/L BPA. Relative to the estimated solvent-control reproduction rates during the main spawning season the rates found here under presence of BPA reflect an induction of less than 10%.
It is strongly recommended for the future, to shorten the experimental time in favour of a better statistical design and the measurement of the reproduction rates, which lead to consistent, time-independent values of the NOEC and ECx, provided the concentration range is chosen appropriately.

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Annex

Annex Table 1: Calculation of the survival rate as exemplified for the Solvent Control. Number of dead snails in replicate a and b (Da, Db) was substracted from the introduced number of 30 specimens, each, at the day when mortality was observed. SCa, SCb: survived number of animals; SC: sum of the survived animals from replicates a and b; SR: Survival rate.

Date	day	Da	Db	SCa	SCb	SC	SR
4.2	1	0	0	30	30	60	1,00
5.2	2	0	0	30	30	60	1,00
6.2	3	0	0	30	30	60	1,00
7.2	4	0	0	30	30	60	1,00
10.2	7	0	0	30	30	60	1,00
11.2	8	0	0	30	30	60	1,00
12.2	9	0	0	30	30	60	1,00
13.2	10	0	0	30	30	60	1,00
14.2	11	0	0	30	30	60	1,00
17.2	14	0	0	30	30	60	1,00
18.2	15	0	0	30	30	60	1,00
19.2	16	0	0	30	30	60	1,00
20.2	17	0	0	30	30	60	1,00
21.2	18	1	0	29	30	59	0,98
24.2	21	0	0	29	30	59	0,98
25.2	22	0	0	29	30	59	0,98
26.2	23	0	0	29	30	59	0,98
27.2	24	0	0	29	30	59	0,98
28.2	25	0	0	29	30	59	0,98
3.3	28	0	0	29	30	59	0,98
4.3	29	0	0	29	30	59	0,98
5.3	30	0	1	29	29	58	0,97
6.3	31	0	0	29	29	58	0,97
7.3	32	0	0	29	29	58	0,97
10.3	35	0	0	29	29	58	0,97
11.3	36	0	0	29	29	58	0,97
12.3	37	1	0	28	29	57	0,95
13.3	38	0	0	28	29	57	0,95
14.3	39	1	0	27	29	56	0,93
17.3	42	0	0	27	29	56	0,93
18.3	43	0	0	27	29	56	0,93
19.3	44	0	0	27	29	56	0,93
20.3	45	0	0	27	29	56	0,93
21.3	46	0	0	27	29	56	0,93
24.3	49	0	0	27	29	56	0,93
25.3	50	0	0	27	29	56	0,93
26.3	51	0	1	27	28	55	0,92
27.3	52	0	0	27	28	55	0,92
28.3	53	0	0	27	28	55	0,92
31.3	56	0	0	27	28	55	0,92
1.4	57	0	0	27	28	55	0,92
2.4	58	0	0	27	28	55	0,92
3.4	59	0	0	27	28	55	0,92
4.4	60	0	0	27	28	55	0,92
7.4	63	0	0	27	28	55	0,92
8.4	64	1	0	26	28	54	0,90
9.4	65	0	0	26	28	54	0,90
10.4	66	0	0	26	28	54	0,90

Annex Table 1 continued

11.4	67	0	0	26	28	54	0,90
14.4	70	0	1	26	27	53	0,88
15.4	71	0	0	26	27	53	0,88
16.4	72	0	0	26	27	53	0,88
17.4	73	0	0	26	27	53	0,88
22.4	78	0	0	26	27	53	0,88
23.4	79	0	0	26	27	53	0,88
24.4	80	0	0	26	27	53	0,88
25.4	81	0	0	26	27	53	0,88
28.4	84	0	0	26	27	53	0,88
29.4	85	0	1	26	26	52	0,87
30.4	86	0	0	26	26	52	0,87
2.5	88	0	0	26	26	52	0,87
5.5	91	0	0	26	26	52	0,87
6.5	92	0	0	26	26	52	0,87
7.5	93	0	0	26	26	52	0,87
8.5	94	0	0	26	26	52	0,87
9.5	95	0	0	26	26	52	0,87
12.5	98	0	1	26	25	51	0,85
13.5	99	0	0	26	25	51	0,85
14.5	100	0	0	26	25	51	0,85
15.5	101	1	0	25	25	50	0,83
16.5	102	0	0	25	25	50	0,83
19.5	105	0	0	25	25	50	0,83
20.5	106	0	0	25	25	50	0,83
21.5	107	0	0	25	25	50	0,83
22.5	108	0	0	25	25	50	0,83
23.5	109	0	0	25	25	50	0,83
26.5	112	0	0	25	25	50	0,83
27.5	113	0	0	25	25	50	0,83
28.5	114	0	1	25	24	49	0,82
30.5	116	0	0	25	24	49	0,82
2.6	119	0	0	25	24	49	0,82
3.6	120	0	0	25	24	49	0,82
4.6	121	0	0	25	24	49	0,82
5.6	122	0	0	25	24	49	0,82
6.6	123	0	0	25	24	49	0,82
10.6	127	0	0	25	24	49	0,82
11.6	128	0	0	25	24	49	0,82
12.6	129	0	0	25	24	49	0,82
13.6	130	0	0	25	24	49	0,82
16.6	133	0	0	25	24	49	0,82
17.6	134	0	0	25	24	49	0,82
18.6	135	0	0	25	24	49	0,82
20.6	137	0	0	25	24	49	0,82
23.6	140	0	0	25	24	49	0,82
24.6	141	0	0	25	24	49	0,82
25.6	142	0	0	25	24	49	0,82
26.6	143	0	0	25	24	49	0,82
27.6	144	0	0	25	24	49	0,82
30.6	147	0	0	25	24	49	0,82
1.7	148	1	0	24	24	48	0,80
2.7	149	0	0	24	24	48	0,80
3.7	150	0	0	24	24	48	0,80
4.7	151	0	0	24	24	48	0,80

Annex Table 2: Calculation of the cumulative number of eggs per female (CNEF) as exemplified for the Solvent Control; the survival data of **Fehler! Verweisquelle konnte nicht gefunden werden.** was used (columns # Survived) to calculate CNEF (# per Survived Fem.) from the cumulative number of eggs (CNE; the female ratio from the bottom of the table was used); a, b: CNE in replicate A and B; SCa, SCb: survived number of animals; SC: sum of the survived animals from replicates a and b.

		20°C SC (solvent control)								
		Cum. #Eggs			**# Survived**			**# per Survived Fem.**		
Date	day	a	b	sum	SCa	SCb	SC	SCa	SCb	mean
4.2	1	76	0	76	30	30	60	4,1	0,0	2,1
5.2	2	142	47	189	30	30	60	7,6	2,7	5,2
6.2	3	142	47	189	30	30	60	7,6	2,7	5,2
7.2	4	168	47	215	30	30	60	9,0	2,7	5,9
10.2	7	268	127	395	30	30	60	14,3	7,3	10,9
11.2	8	403	127	530	30	30	60	21,5	7,3	14,6
12.2	9	606	332	938	30	30	60	32,3	19,0	25,9
13.2	10	660	460	1120	30	30	60	35,2	26,3	30,9
…	…	…	…	…	…	...	…	…	…	…
23.6	140	6960	6794	13754	25	24	49	445,4	485,3	464,6
24.6	141	7095	6901	13996	25	24	49	454,1	492,9	472,8
25.6	142	7095	6901	13996	25	24	49	454,1	492,9	472,8
26.6	143	7155	7003	14158	25	24	49	457,9	500,2	478,2
27.6	144	7278	7106	14384	25	24	49	465,8	507,6	485,9
30.6	147	7335	7106	14441	25	24	49	469,4	507,6	487,8
1.7	148	7478	7225	14703	24	24	48	498,5	516,1	507,0
2.7	149	7478	7239	14717	24	24	48	498,5	517,1	507,5
3.7	150	7679	7351	15030	24	24	48	511,9	525,1	518,3
4.7	151	7778	7559	15337	24	24	48	518,5	539,9	528,9
start no. of animals										
		30	30	60						
surviving animals										
female no.		15	14	29						
male no.		9	10	19						
female ratio		0,625	0,583	0,604						
mortality {%}		20%	20%	20%						
eggs per female										
		518,5	539,9	528,9						
dead animals found on day										
		17	30							
		30	51							
		32	70							
		64	85							
		101	98							
		148	114							
	n:	**6**	**6**							

Annex Table 3: Results of the probit analysis conducted for mortality (survival) over time as being observed under increasing concentrations of BPA
Survival (S) and percent mortality (%M) as computed from the raw data for test intervals selected; ECxx: effect levels as selected; lower 95%-cl, upper 95%-cl: lower and upper 95%-confidence limits.; *pm: Probit analysis using linear max. likelihood regression.

Treatment	**0-21 h**		**0-30 h**		**0-50 h**		**0-73 h**		**0-100 h**		**0-127 h**		**0-151 h**	
[µg/L]	**S**	**%M**	**S**	**%M**	**S**	**%M**	**S**	**%M**	**S**	**%M**	**S**	**%M**	**S**	**%M**
SC	59	1,7	58	3,3	56	6,7	53	11,7	51	15,0	49	18,3	48	20,0
0,25	59	1,7	58	3,3	53	11,7	49	18,3	43	28,3	41	31,7	40	33,3
0,50	55	8,3	52	13,3	46	23,3	44	26,7	42	30,0	41	31,7	39	35,0
1,00	58	3,3	57	5,0	52	13,3	50	16,7	44	26,7	42	30,0	40	33,3
5,00	59	1,7	56	6,7	52	13,3	51	15,0	44	26,7	43	28,3	39	35,0
EC10		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.
lower 95%-cl		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.
upper 95%-cl		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.
EC20		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	86,28	*pm
lower 95%-cl		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.
upper 95%-cl		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.
EC50		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.
lower 95%-cl		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.
upper 95%-cl		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.
p(F)(slope = 0)		0,370		0,014*)		0,187		0,570		0,338		0,031*)		0,516

*) Slope either < zero or too high variability to compute ECx in the observed concentration range