QUANTICS Biostatistics

Syngenta Ltd

Chronic study in the fathead minnow. Numbers of eggs per surviving female per day: NOEC and EC_{10}

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Section 1: Introduction

A chronic study in the fathead minnow with chlorothalonil was conducted in 1980. Syngenta now require statistical analysis of a study endpoint that was not originally reported: the number of eggs per surviving female per day. Both the No Observed Effect Concentration (NOEC) and the EC10, plus a 95% confidence interval, are required.

Section 2: Data

Syngenta has provided Quantics with an excel data file containing the relevant data, shown in Table 1.

Table 1: Data provided by Syngenta

Treatment µg/L		Eggs	Surviving females	Days	Eggs per surviving female	Eggs per surviving female per day
Dilution water control	A	2265	5	111	453	4.08
	B	3660	6	111	610	5.50
Solvent control	A	2332	6	111	389	3.50
	B	1339	6	111	223	2.01
0.6	A	3564	6	111	594	5.35
	B	301	6	111	50	0.45
1.4	A	1868	4	111	467	4.21
	B	3266	6	111	544	4.90
3.0	A	713	4	111	178	1.61
	B	111	3	111	37	0.33
6.5	A	12	6	111	2	0.02
	B	676	6	111	113	1.02
16.0	A	18	2	111	9	0.08
	B	0	0	111	-	-

Section 3: Statistical methods

3.1 Guidances

The relevant regulatory methods are described in OECD Series on Testing and Assessment, Number 54: Current approaches in the statistical analysis of ecotoxicity data: guidance to application (2006) [1] and the EFSA Technical Report Outcome of the pesticides peer review meeting on general recurring issues in ecotoxicology (2015) [2].

3.2 Control groups

The study included two control groups: dilution water control and solvent control. In each analysis the control groups were included individually and pooled.

3.3 Outliers

The data were examined graphically for outliers.

3.4 Estimation of ECx

For the estimation of the EC10 and EC20, a concentration - response model must be fitted to the data [1]: y = f(x). The recommended procedure was followed:

- Fit various models.
- Choose best fitting model(s).
- Estimate ECx and 95% confidence interval.
- Report the lowest of the lower confidence bounds.

The Hill model (paragraph 317, [1]), with x = ln(concentration) was deemed an appropriately flexible model for the current data set because it was expected that the response would be monotonic across the concentrations:

$$y = y(0) + [y(\infty) - y(0)]\frac{x^n}{EC_{50}^n + x^n}$$

The model was fitted in two ways: estimating $y(\infty)$ and fixing $y(\infty)$ at zero.

The models were estimated using ordinary least squares via the Levenberg-Marquardt algorithm.

Both the EC10 and the EC20 ([1], [2]) were estimated from the models fits and their 95% confidence intervals were calculated using the Delta method [3].

3.5 Estimation of NOEC

3.5.1 Parametric analysis

The two control groups were compared using a t test. (There were not enough degrees of freedom to check the distributional assumptions.)

For each control group separately, and for both control groups together, the treated groups were compared pairwise with the control group as follows:

- For both control groups together, the data were checked for homogeneity of variance using Levene's test [4]. (Levene's test is not valid when no group has more than 2 responses.) With so few responses per concentration, tests for normality lack power; therefore normality was not tested.
- Where the variances were homogeneous, analysis of variance was conducted, followed by a one-sided Dunnett's test [5] at the 5% level to compare each treatment group with the control group.
- Where the variances were not homogeneous, the groups were examined for differences. Outliers were omitted if appropriate and the process repeated. Where it was not possible to achieve homogeneity of variance, Wilcoxon tests were used to compare treatment groups with control groups.
- The Lowest Observed Effect Concentration (LOEC) was estimated as the lowest concentration showing a significantly lower number of eggs per surviving female per day than the control group. The NOEC was estimated as the tested concentration below the LOEC.

3.5.2 Non-parametric analysis

The two control groups were compared using the Wilcoxon test.

For each control group individually, and for pooled control groups, the treated groups were compared pairwise with the control group as follows [1]:

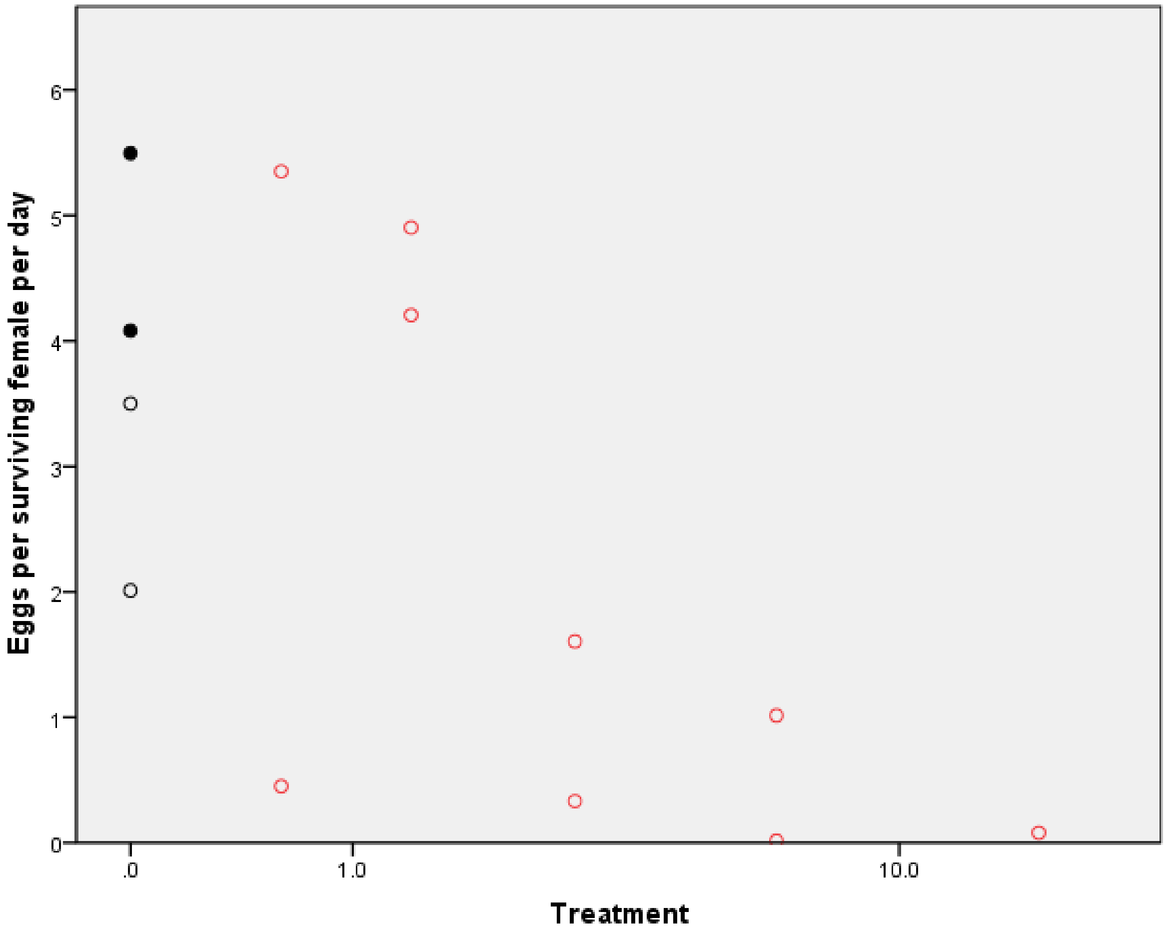
- The data were checked for homogeneity of variance using Levene's test [4].
- With so few responses per concentration, tests for normality lack power; therefore normality was not tested.
- If the variances were homogeneous, analysis of variance was conducted, followed by a one-sided Williams' test [5] at the 5% level to compare each treatment group with the control group.
- Where the variances were not homogeneous, the Jonckheere Trend Test [6] was used stepwise at the 5% level to compare each treatment group with control.
- The Lowest Observed Effect Concentration (LOEC) was estimated as the lowest concentration showing a significantly lower number of eggs per surviving female per day than the control group. The NOEC was estimated as the tested concentration below the LOEC.

Section 4: Results

4.1 Data summary

Figure 1 shows the response data plotted against the concentration. The results at 0.6 µg/L are clearly more different from each other than in the other groups but with only two data points it is not possible to say whether either of these is an outlier.

Figure 1: Eggs per surviving female per day



Solid black circles: Dilution Water Control
Hollow black circles: Solvent Control
Hollow red circles: Treated groups

4.2 Estimation of ECx

Attempts were made to fit the two Hill models to the data with (a) Controls included individually and pooled, (b) including and excluding the lower data point at 0.6 µg/L and (c) including and excluding both data points at 0.6 µg/L.

In all cases where either the Solvent Control data or the lower data point at 0.6 µg/L were included, the model fit was singular in the sense that the slope was infinite and no fit was possible.

Figures 2 and 3 show the two model fits with the Solvent Control data and the lower data point at 0.6 µg/L omitted: with y(∞) estimated and with y(∞) set to zero respectively. These models were also fitted excluding both the Solvent Control group and the 0.6 µg/L treatment group.

Table 2 shows the parameters estimates for all four models. Confidence intervals are included for the EC10 and the EC20. The model fits are very similar. The confidence intervals are wider when the whole group at 0.6 µg/L was omitted.

Figure 2: Hill model with y(∞) estimated. Dilution Water Control only; lower data point at 0.6 µg/L omitted

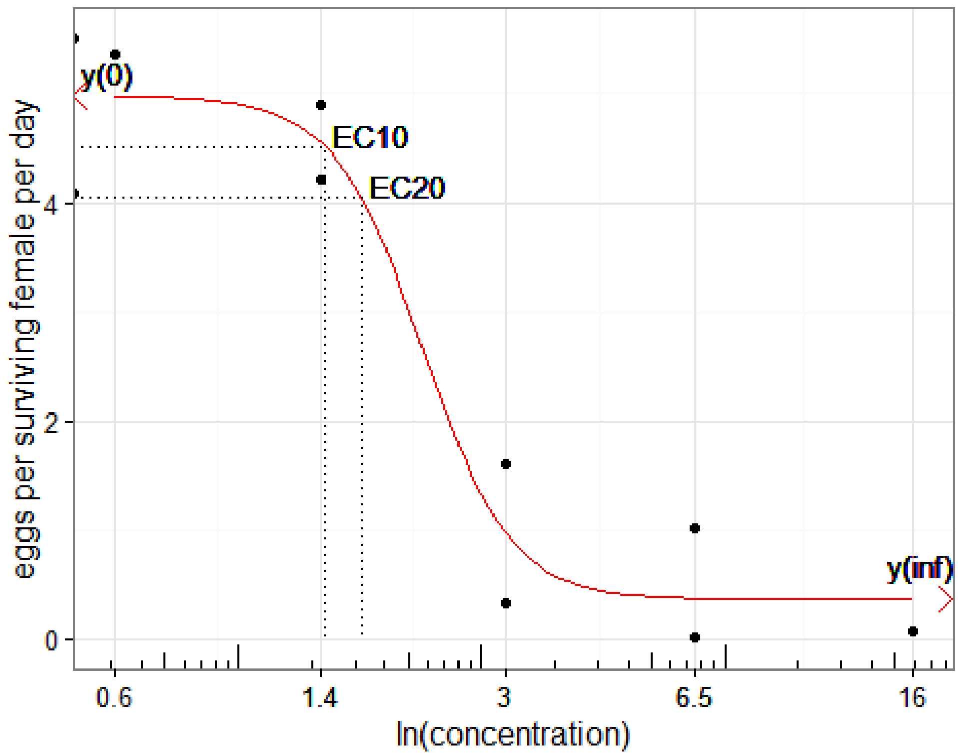


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Figure 3: Hill model with y(∞) fixed at 0. Dilution Water Control only; lower data point at 0.6 µg/L omitted

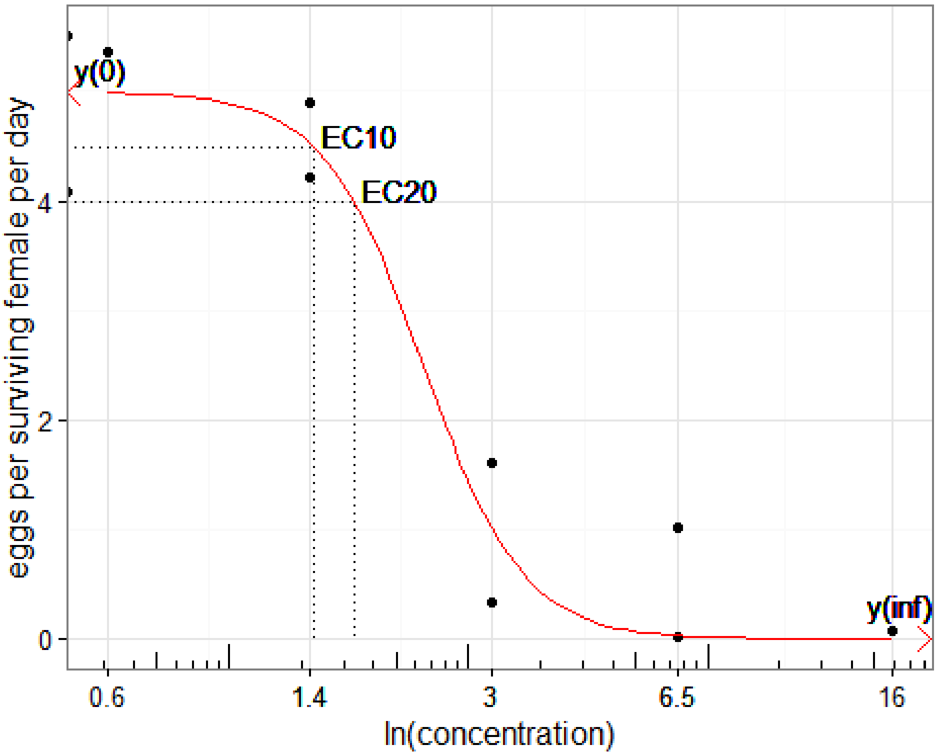

Table 2: Estimates of EC10 and EC20: Solvent Control group omitted

	Lower data point at 0.6 µg/L omitted		Both data points at lower data point at 0.6 µg/L omitted	
Model	Unconstrained	Constrained	Unconstrained	Constrained
y(∞)	0.364	0	0.367	0
n	-5.483	-4.790	-6.154	-5.312
EC_{50}	0.755	0.811	0.799	0.845
y(0)	4.977	4.994	4.793	4.821
Residual sum of squares	2.90	3.27	2.69	3.08
EC_{10}	1.43	1.42	1.56	1.54
Upper 95% bound for EC_{10}	2.88	2.85	4.81	4.13
Lower 95% bound for EC_{10}	0.71	0.71	0.50	0.57
EC_{20}	1.65	1.68	1.78	1.79
Upper 95% bound for EC_{20}	2.96	2.92	4.47	3.90
Lower 95% bound for EC_{20}	0.92	0.97	0.70	0.82

4.3 Estimation of NOEC

4.3.3 Parametric analysis

The control groups were not significantly different (p = 0.19, t test).

The variances amongst the groups were significantly different when the control groups were included together (p = 0.035, Levene's test). This heterogeneity appeared to be caused by the lower data point for treatment group 0.6 µg/L: when this group was excluded the variances were homogeneous (p = 0.587).

The comparison of groups was conducted using ANOVA but excluding the lower data point in treatment group 0.6 µg/L. There were then two groups with only one observation each. The pairwise comparisons with Control were not conducted for these groups. The results are shown in Table 3.

Table 3: Comparison of groups via ANOVA

Treatment	n	Mean Eggs per surviving female per day	Difference in means, Dunnett's test *		
			Control (both)	Dilution Water Control	Solvent Control
Control (both)	4	3.77			
Dilution Water Control	2	4.79			
Solvent Control	2	2.76			
0.6 µg/L **	1	5.35			
1.4 µg/L	2	4.56	0.78	-0.23	1.80
3.0 µg/L	2	0.97	-2.80 *	-3.82 *	-1.79
6.5 µg/L	2	0.52	-3.26 *	-4.27 *	-2.24
16.0 µg/L	1	0.08			
Overall p (ANOVA)			0.022	0.012	0.021
LOEC			3.0	3.0	> 6.5
NOEC			1.4	1.4	≥ 6.5

* one sided p <0.05
** Outlier excluded

To compare treatment group 0.6 µg/L with the control groups, both separately and combined, Wilcoxon tests were conducted. In all three cases there was no significant difference (P > 0.05).

It was not deemed necessary to compare the highest concentration (16 µg/L) with the control groups, as there was a clear effect, and also the lower concentrations had already identified a LOEC in all cases except the comparison with Solvent Control.

4.3.4 Non-parametric analysis

The control groups were not significantly different ($p = 0.33$, Wilcoxon test).

The variances amongst the groups were significantly different when the control groups were included together ($p = 0.035$, Levene's test) or separately ($p = < 0.001$, Solvent Control only; $p = < 0.001$, Dilution Water Control only).

The dose groups were compared with the controls using the Jonckheere Trend test. The results are shown in Table 4.

For comparisons with both the pooled controls and dilution water control there was a significant trend towards decreasing number of eggs per female ($p \le 0.05$) from control up to a concentration of 6.5 µg/L and up to 16.0 µg/L. For comparisons with the solvent control there was only a significant trend towards decreasing number of eggs per female ($p \le 0.05$) from control up to the highest dose of 16.0 µg/L.

Table 4: Comparison of groups via Jonckheere Trend test

Treatment	n	Median Eggs per surviving female per day	Difference in medians		
			Pooled Controls	Dilution Water Control	Solvent Control
Pooled Controls	4	3.79			
Dilution Water Control	2	4.79			
Solvent Control	2	2.76			
0.6 µg/L	2	2.90	-0.89	-1.89	0.14
1.4 µg/L	2	4.56	0.77	-0.23	1.80
3.0 µg/L	2	0.97	-2.82	-3.82	-1.79
6.5 µg/L	2	0.52	-3.27 *	-4.27 *	-2.24
16.0 µg/L	1	0.08	-3.71 *	-4.71 *	-2.68 *
LOEC			6.5 µg/L	6.5 µg/L	16.0 µg/L
NOEC			3.0 µg/L	3.0 µg/L	6.5 µg/L

* Significant trend over concentrations up to this one; $p \le 0.05$, Jonckheere Trend Test

Section 5: Conclusions

For the concentration-response modelling, the data presents some challenges. It was not possible to fit the Hill model (a fairly general monotonic concentration-response model) when the Solvent Control data were included. To achieve a model fit it was necessary to remove the Solvent Control group and also the lower data point at 0.6 µg/L. The EC10 was estimated to be between 1.42 µg/L and 1.56 µg/L; the lower 95% confidence bound for the EC10 ranged from 0.50 µg/L to 0.71 µg/L.

NOEC: Parametric analysis

The lower data point at 0.6 µg/L also affected the analysis of variance required to estimate the NOEC. It was omitted from the analysis in order to achieve variance homogeneity. With only one data point in each of two groups (0.6 µg/L and 16.0 µg/L) it was possible to conduct the ANOVA but the pairwise comparisons with control were not possible for those two groups.

For the comparison with Solvent Control alone, there were no statistically significant differences. Thus the LOEC was greater than 6.5 µg/L and the NOEC could only be said to be at least 6.5 µg/L.

However the NOEC with respect to the Dilution Water Control alone, and with respect to the combined controls, was 1.4 µg/L

When the lower data point at 0.6 µg/L was included in the analysis and the 0.6 µg/L group was compared directly with the Control groups both separately and combined (via the Wilcoxon test), there was no significant difference. Thus the above estimates of the NOEC were unaffected by the anomalous data in the 0.6 µg/L group.

NOEC: Non-parametric analysis

The NOEC was also estimated using a non-parametric approach, via the Jonckheere Trend test. The estimated value was 3.0 µg/L in the Dilution Water control group was used, wither alone or pooled with the Solvent Control group. If the Solvent Control group alone was used, then the NOEC was estimated to be 6.5 µg/L.

References

1 OECD Series on Testing and Assessment, Number 54: Current approaches in the statistical analysis of ecotoxicity data: guidance to application (2006)

2 EFSA Technical Report Outcome of the pesticides peer review meeting on general recurring issues in ecotoxicology (2015)

3 Oehlert, G. W. (1992), A Note on the Delta Method, The American Statistician, Vol. 46, No. 1, p. 27-29

4 Levene, H. (1960). In Contributions to Probability and Statistics: Essays in Honor of Harold Hotelling, I. Olkin et al. eds., Stanford University Press, pp. 278-292.

5 Williams DA (1971). A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics 27, 103-117.

6 Jonckheere, A. R. (1954). A distribution-free k-sample test against ordered alternatives. Biometrika. 41: 133–145