

# Memorandum

**To:** Mr. Mark Odom and Dr. Ramón Leon  
**From:** Dawn Heaney and Yuki Taketani  
**Date:** February 21, 1997  
**Re:** Fish Embryo Analysis

---

## Summary

A study was done on the effects of heavy metal toxicity on embryonic development of fish (Medaka) embryos. The embryos were divided into 6 different exposure groups, and embryos in each group were cultured individually with 1 ml Nickel Chloride solutions. The concentrations ranged from 0ppm to 70ppm (0, 10, 20, 30, 50, 70). Individual embryos were incubated in 1.0 cm plastic microwell plates at 15 degrees Celsius. The embryonic development of each given individual was tracked for 29 days, and hatching time was recorded. The embryos were classified as censored if they did not hatch or if they died before experiment was terminated.

Our main study questions were the following:

- To determine the lowest Nickel Chloride concentration which produces a significant effect on embryo's hatching time compared to the control group
- To construct a model for future prediction for hatchday
- To construct a model for mortality of embryos.

## Conclusions

- The LogNormal model assuming equal slopes for each NiCl concentration level can be used for future predictions of hatching time.
- Median hatching time increases as the concentration of NiCl increases.
- Fish embryos cultured with 30 ppm and 50 ppm NiCl hatch significantly later than the control group (0 ppm).
- Predicted LC50 (Lowest concentration which causes 50% of mortality) for NiCl is 33.46 ppm.

## Summary

A study was done on the effects of heavy metal toxicity on the embryonic development of fish (Medaka) embryos. The embryos were divided into 6 different exposure groups and were cultured individually with 1 ml Nickel Chloride solutions. The concentrations ranged from 0ppm to 70ppm (0, 10, 20, 30, 50, 70). Individual embryos were incubated in 1.0 cm plastic microwell plates at 15 degrees Celsius. The embryonic development of each given individual was tracked for 29 days and hatching time was recorded. The embryos were classified as censored if they did not hatch by the 29th day or if they died before the experiment was terminated.

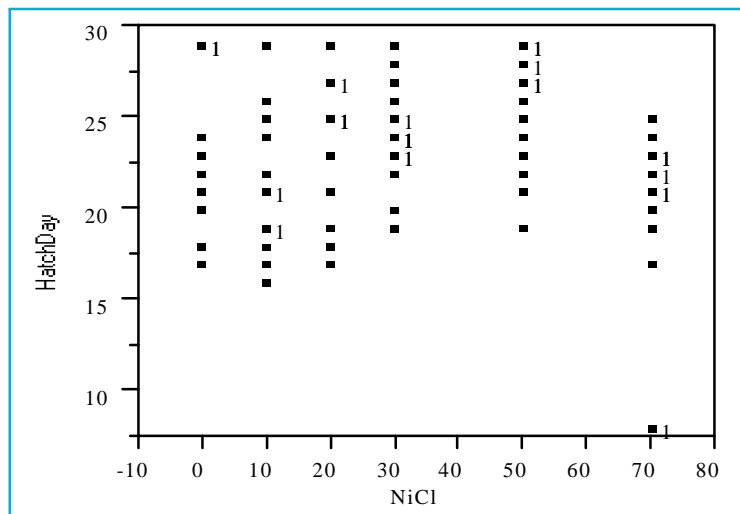
## Conclusions

- The LogNormal model assuming equal slopes for each NiCl concentration level can be used for future predictions of hatching time.
- Median hatching time increases as the concentration of NiCl increases.
- Fish embryos cultured with 30 ppm and 50 ppm NiCl hatch significantly later than the control group (0 ppm).
- Predicted LC50 (Lowest concentration which causes 50% of mortality) for NiCl is 33.46 ppm.

## Analysis

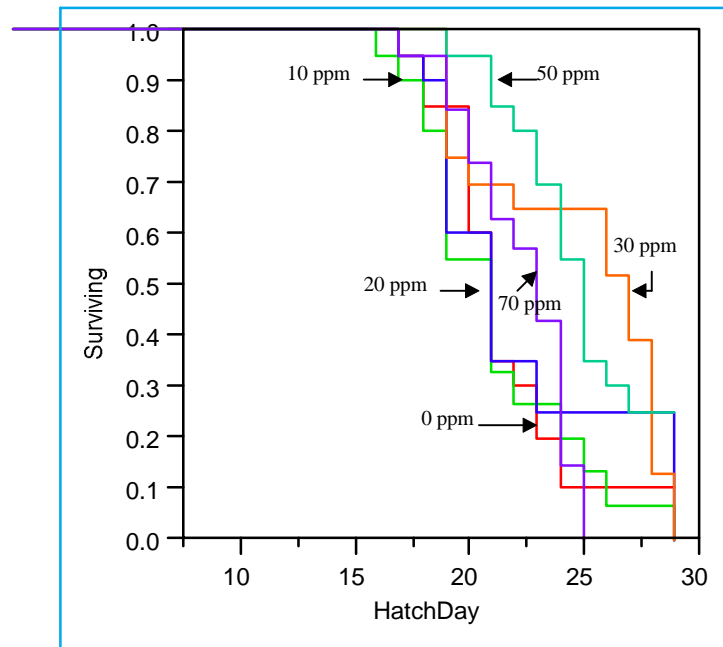
### Scatter Plot and Survival Plot of the Raw Data

The following plot shows a graphical representation of the data by NiCl concentration level. It shows that no embryos hatched before the 15th day (Figure 1). Note that the experiment was only conducted for 29 days so any embryos that had not hatched by this date were considered hatched on the 29th day, but were called censored in the experiment. They were also considered censored if they died before hatching.



**Figure 1.** Plot of Hatch Day by Concentration Group. The points labeled 1 indicate a censoring time.

Figure 1 shows that no hatching is observed before the 15th day. The survival plot in Figure 2 confirms this fact showing this probability equal to one. From that day on, the probability of not hatching decreases (hatching increases) almost linearly for each concentration group.

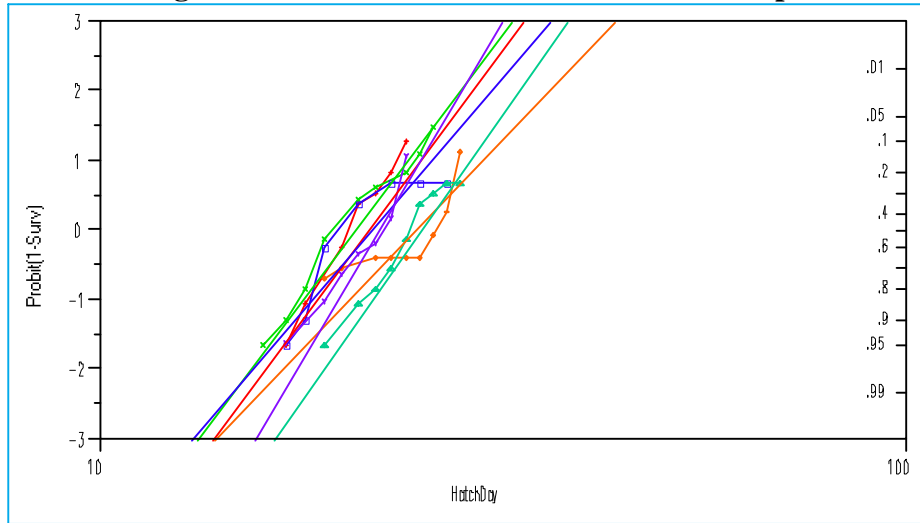


**Figure 2.** This survival plot shows the probability of not hatching along the vertical axis and the day of hatching along the horizontal axis. The different lines are the individual NiCl concentration levels.

## Model Fitting

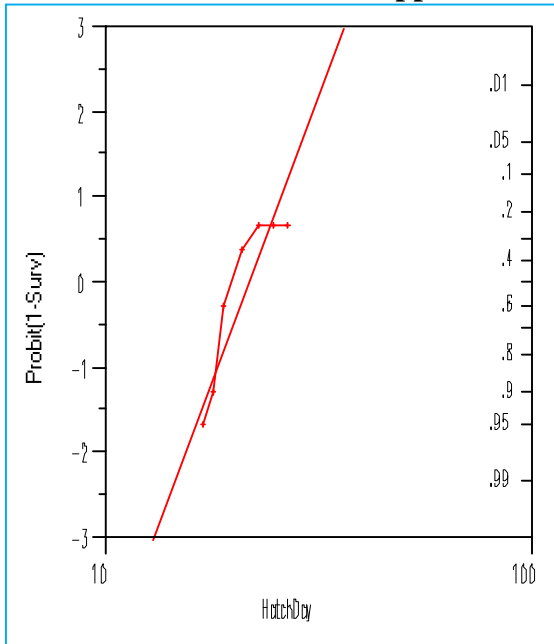
In order to do further analysis, it is important to see what type of model fits the data. The following plot shows each of the concentration groups fit with a LogNormal model (Figure 3). This model appears to fit appropriately for four of the six concentration groups. Concentration levels 20 and 30, however, do not seem to fit this model as well (Figures 4 & 5). It will be seen later that this is not a problem.

### LogNormal Plot of All Six Concentration Groups



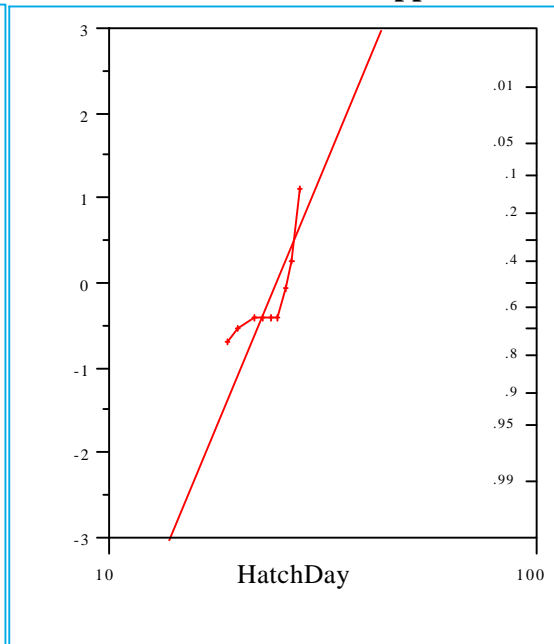
**Figure 3.** Plot of a LogNormal model fit to each concentration level.

### Concentration 20ppm



**Figure 4.** Plot of a LogNormal model fit to concentration level 20ppm.

### Concentration 30ppm

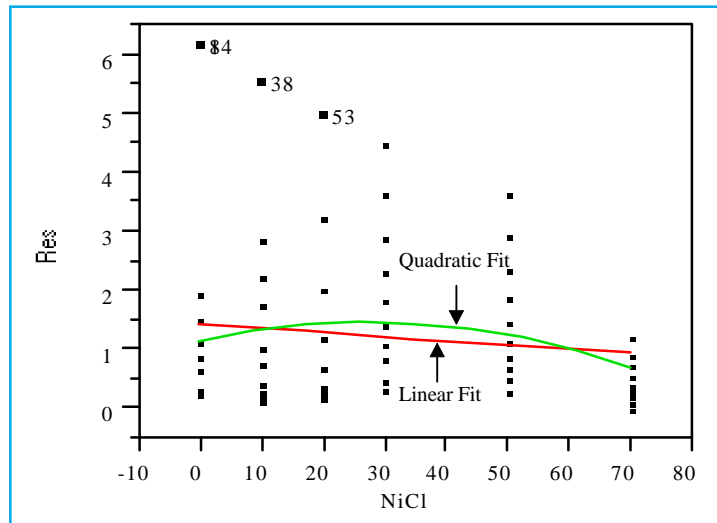


**Figure 5.** Plot of a LogNormal model fit to concentration level 30 ppm.

Although there appears to be a discrepancy in the LogNormal fit above, the model is fit to all of the data combined. This assumes that the increase in the number of days to hatching is the same for each level of NiCl.

## Residual Analysis

After a LogNormal model is fit to the data, residuals should be checked for possible errors in the model or outlying points. We can see from the following residual plot that it is possible to fit a quadratic term of NiCl to the model (in addition to the linear term), but it is not necessary. The two lines look very similar and at this point there is no need to complicate the model. We can also see that four points (8, 14, 38, 53 as indicated in Figure 6 below) appear to stand out from the rest of the data. They will be eliminated from the remainder of the analysis.



**Figure 6.** Plot of residuals of LogNormal fit of the data. The straight line represents the linear model in NiCl (that is assumed above). The slightly curved line represents the second order model in NiCl. The labeled points are considered outliers.

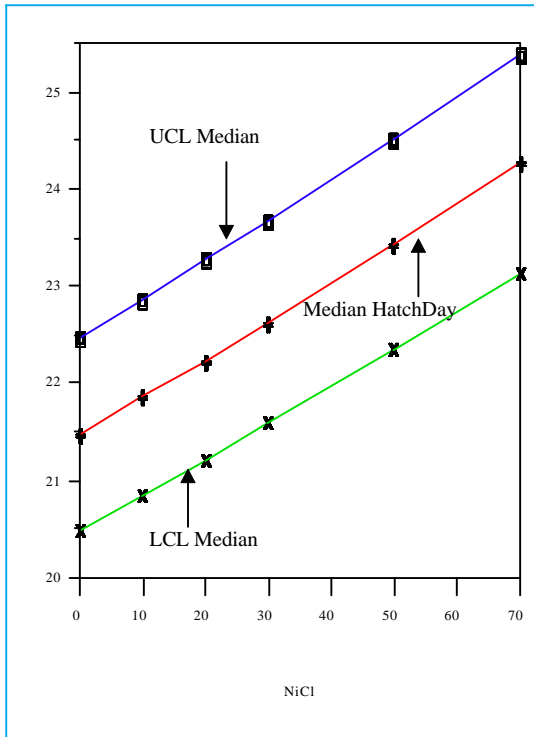
## Comparison of Median Hatching Time

One of the important things to know is the median hatching time for each of the six concentration groups. The following table shows the median hatchday based on the LogNormal model with all of the groups included (Table 1).

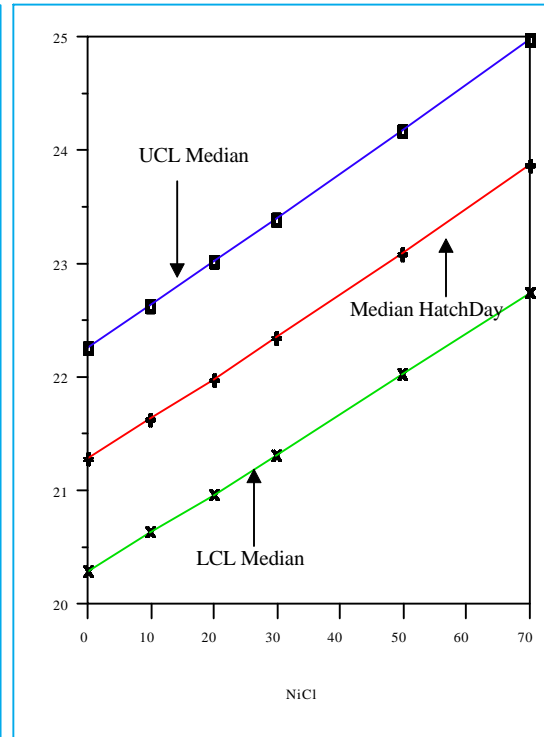
NiCl Concentration	Median Hatchday
0	21.49026
10	21.86747
20	22.25129
30	22.64185
50	23.44366
70	24.27386

**Table 1.** Median Hatch Day for each of the 6 NiCl Concentration Groups

Although it is important to know the median Hatch Day for each of the concentration levels, it is also important to have confidence limits on these numbers. Figure 7 is a plot of the median Hatch Day for each concentration level along with the 95% confidence limits. This means that you are 95% confident that the median hatch day for embryos is within the lines (given the specific concentration). Figure 8 is the same type of plot, but is based on the LogNormal model with NiCl groups 20 and 30ppm excluded. As we can see, the two plots are very similar and thus it is not a problem that originally these two groups did not seem to fit the LogNormal model very well (note that the scale of the two plots is slightly different).



**Figure 7.** Plot of the median hatch day for each level of NiCl with all 6 levels included in the analysis.



**Figure 8.** Plot of the median hatch day for each level of NiCl with groups 20 and 30 excluded from the analysis.

## Analysis of Accelerated Life Test

Up to this point the assumption of equal slopes for each NiCl level has been made. This model is typically called M1 and is described in more detail in the Appendix. It was of interest to see if there was a significant difference between this model and the model which assumes different slopes for each NiCl level (model M2). A significant difference was not found between these two models, thus our assumption of equal slopes is sufficient. It was also of interest to see if the model that is assumed is significantly different from a model assuming separate distributions for each concentration level (model M3). The following table shows the -log likelihood for the model that is assumed (M1) and these values for each of the individual NiCl levels (M3, Table 2). The sum of the -log likelihood of all six concentrations is -21.223. Using a  $\chi^2$  test, the

hypothesis that all concentrations have common sigma is accepted (see Appendix). Thus the model that we have assumed for the plots is sufficient.

Model	NiCl Concentration	df	-Log Likelihood
M1	Combined	3	-12.767
M3	0 PPM	2	-6.046
	10 PPM	2	-7.425
	20 PPM	2	-1.415
	30 PPM	2	2.121
	50 PPM	2	-3.248
	70 PPM	2	-5.210
	Total	12	-21.223

**Table 2.** -Log Likelihood values for each type of model that is tested.

### Comparison Between Control (0ppm) and Other Concentration Groups

The previous survival analysis indicates that there are significant differences in hatchday among the six NiCl concentration groups with p-value < .10 (Table 3). The definitions of the two tests given below are described in detail in the Appendix.

Test	Chi-Square	df	p-value
Log-Rank	12.4969	5	0.0127
Wilcoxon	18.6560	5	0.0022

**Table 3.** Overall comparison test of NiCl Concentration levels.

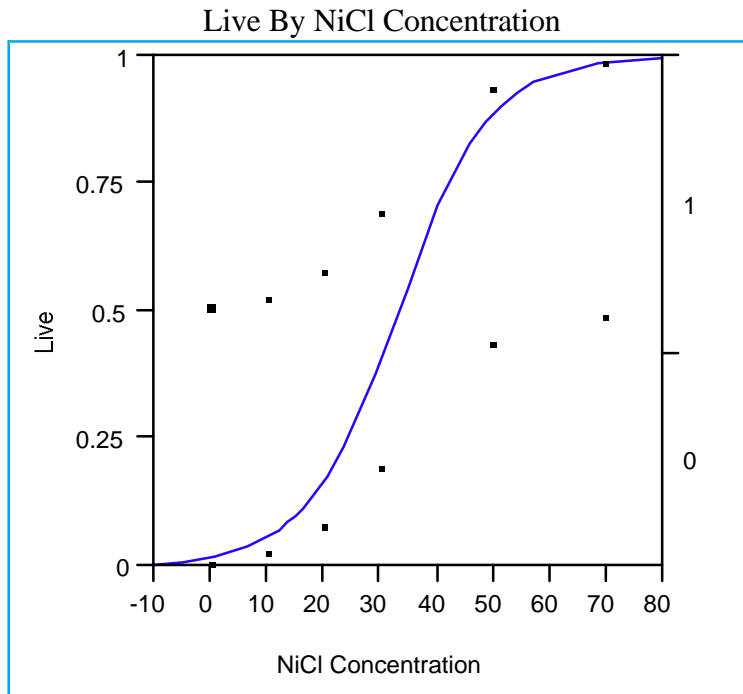
Further analysis of individual comparisons reveals that 30 ppm and 50 ppm groups have significantly different hatching time from the control group (0ppm), using an alpha level of 0.10 (Table 4).

NiCl Concentration	Test	Chi-Square	df	p-value
10 PPM	Log-Rank	0.3748	1	0.5404
	Wilcoxon	0.5277	1	0.4676
20 PPM	Log-Rank	0.0848	1	0.7709
	Wilcoxon	0.0068	1	0.9345
30 PPM	Log-Rank	3.6585	1	0.0558
	Wilcoxon	4.3083	1	0.0379
50 PPM	Log-Rank	8.5920	1	0.0034
	Wilcoxon	11.6925	1	0.0006
70 PPM	Log-Rank	1.1466	1	0.2843
	Wilcoxon	1.9424	1	0.1634

**Table 4.** Individual comparisons for 5 NiCl concentration groups versus the control group (0ppm).

## Mortality Rate Estimate of Embryos

To investigate the mortality rate of embryos, logistic regression was conducted. The following is a non-linear plot of live days by NiCl concentration. LC50 (Lowest Concentration which causes 50% of mortality) of NiCl was determined to be 33.46 ppm. The lower and upper 95% limits on this value are 28.5908200ppm and 39.4607238ppm, respectively.



**Figure 9.** Plot of mortality rate by NiCl concentration level.

## Appendix: Data Analysis

- LogNormal Model:

$$f(t) = (2\pi\delta^2t^2)^{-1/2} \exp \{-(\log t - \mu)/(2\delta^2)\}$$

Using the LogNormal model above, the formula for calculating residuals for a LogNormal model is :

$$\exp\left(\frac{\ln Hatchday - (3.07 + 0.0017 \cdot NiCl)}{0.165}\right)$$

- Accelerated Life Test

M1 is a model that assumes a common sigma and a common slope for all concentration levels. For this model, -log likelihood is -12.767.

M2 is a model that assumes a common sigma, but different slopes for each concentration level.

M3 is a model that assumes separate LogNormal distributions for all the concentration levels 0, 10, 20, 30, 50, and 70 ppm. The -log likelihood value is -21.223.

The  $\chi^2$  test that is conducted to test for a significant difference between models M1 and M3 is:

$$2*[-21.223 - (-12.77)] = 16.906 < 16.919 \chi^2_{.05, 12-3},$$

thus as stated in the paper, the assumption of equal slopes for each concentration level is sufficient.

The following table contains the parameter estimates for the combined LogNormal model and the estimates for each concentration level assuming a different LogNormal distribution for each.

**Parameter Estimates**

NiCl Concentration	Intercept	Sigma
Combined	3.068	0.165
0 PPM	3.069	0.148
10 PPM	3.033	0.150
20 PPM	3.079	0.171
30 PPM	3.204	0.191
50 PPM	3.221	0.139
70 PPM	3.100	0.118

Thus we can see why our original model of assuming common sigma and common slope was appropriate.

- Test for Differences Among Groups

Log-Rank Test

It places more weight on larger survival times and is most useful when the ratio of hazard function in the groups being compared is approximately constant.

Wilcoxon Test

It places more weight on early survival times and is the optimum rank test if the error distribution is logistic.

- Logistic Regression

For mortality of fish embryo, the Logistic Regression model to find the probability of dying is

$$P(\text{NiCl}) = -4.084 + \frac{1}{1 + \exp(-\text{NiCl} * 0.122)}$$