Simply Probits

Probit analysis for lethal dose and growth inhibition calculations

Simply Probits

1. Introduction

Simply Probits has been designed for users of PCs running Windows 95 or 98 who wish to estimate lethal or environmental concentrations using toxicity test data. It has been designed to meet the needs of laboratories undertaking standard toxicity tests for industry. The program has been designed to be quick and intuitive to use and to comply with Good Laboratory Practice guidelines for the use of computerised systems in testing laboratories. **Simply Probits** offers two types of analysis. The first, termed below Lethal Dose calculations, performs a typical probit analysis on the pattern of survival of a series of experimental groups of organisms exposed to a range of concentrations of a test substance. The procedure undertaken conforms to ISO/DIS 14669. The second, termed below growth analysis, undertakes analysis of a test to study the inhibition of the growth of test cultures of organisms such as algae that are exposed to different levels of the test compound. The analysis and presentation of the results conforms to ISO 10253.

Below we give a brief introduction to how to use *Simply Probits* and the computational methods used to estimate the parameters. Once the program has been installed, most users will find it convenient to use the context sensitive help.

2. Installation

System requirements:
1. An IBM 486 or Pentium PC.
2. 1 MB disk space
3. SVGA screen
4. Windows 95 or 98 operating system.

To install:
1. Place the Simply Probits floppy disc in the drive.
2. From the Start button choose Run.
3. Click on the browse button, select the floppy drive and select setup.exe
4. Click OK and Follow the instructions given by the install program.

When installation is complete there will be a folder (directory) on your hard disk which holds the **Simply Probits** and an icon on the start menu.

Uninstalling:
An uninstall program which is placed by the installation program in the same directory as the **Simply Probits** program files can be used to remove the program from your hard disk. This program can also be found on the start menu.

3. Experimental design

At the outset it must be noted that you can only obtain a reliable estimate using probit analysis if the experiment is run over a range of concentrations or conditions which range in their effect from negligible (the controls) to a level greater than that which would be considered significant. Thus if an animal is exposed to a range of concentrations of a potentially lethal chemical such that at the maximum dose used only 5% of the test organisms died, it would be inappropriate to estimate an LD50 (Lethal dose at which 50% of animals would be expected to die). Conversely, an LD50 cannot be estimated if the animals die at all the test concentrations. In this case we can only state that the LD50 value lies between zero (the control level) and the first concentration used and you do not need a program to reach this conclusion. Thus the first stage must be to undertake a 'sighting' experiment to determine the concentrations over which the final experiment should be undertaken.

Both Lethal dose calculations and growth analysis procedures available in Simply Probits assume that the experimental protocols are each of a set type that is summarised below.

Lethal dose experimental protocol

The organisms are divided into groups each holding the same number of individuals. Each group is then exposed to a known concentration of the test substance with one group, the control, not exposed to the test substance. There should, wherever possible, be the same number of replicate groups at each concentration. There are often more controls, as losses in control groups, possibly caused by random chance or bad handling, can seriously distort the analysis. The exposure is carried out for a set period of time (say 48 hours) and the number of individuals alive in each test group counted.

Thus the set up in a typical protocol in which groups of 5 animals are used can be summarised in a table as:

Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
Control	5	5	5	5	5
0.1 mg/l	5	5	5	5	5
1 mg/l	5	5	5	5	5
5 mg/l	5	5	5	5	5
10 mg/l	5	5	5	5	5

And the number alive 48 hours later as:

Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
Control	5	5	5	5	5
0.1 mg/l	4	5	5	5	4
1 mg/l	4	4	4	4	5
5 mg/l	2	3	1	2	2
10 mg/l	0	1	2	0	1

When you choose *New* from the file menu Simply Probits opens a form in which you specify the initial structure of your experiment. To set up a lethal dose experimental data set first click on the *Lethal dose calculation* in the *Type of analysis* panel. If the same numbers of animals are used in each group then check that *Same starting levels* is ticked and type this number in the starting level box. In the *Time Step* box enter the time period over which the test was undertaken. Now specify the number of replicates and the number of test concentrations used. Finally, type in the lower right hand box the actual concentrations used.

Now click on Ok and the data grid will open with the experimental design you have specified laid out ready for you to enter the experimental results

Growth analysis protocol

In this type of experiment the objective is to estimate the concentration of a substance that produces a set amount of growth inhibition in a culture of an organism such as a single celled alga. It can happen that rather than observing inhibition the test cultures actually grow faster than the controls! If this occurs you will see a message warning that the experiment has produced negative inhibition and pointing out that a probit analysis is not possible. Having clicked on OK you will still be able to see plots of the growth rate and intermediate calculations.

A number of cultures normally holding approximately the same concentration of the organism are set up. Each of these cultures is then exposed to a known concentration of the test substance with some, the controls, not exposed to the test substance. There should, wherever possible, be the same number of replicate cultures at each concentration. The number of control replicates can be different from the number used for the test concentrations. It is often larger, as poor growth in control cultures, possibly caused by

random chance or bad handling, can seriously distort the analysis. The cultures are then allowed to develop and the concentration of organisms present measured at set time periods.

Thus a typical table of results expressed in cells/ml would be as follows:

Concentration	Time	Replicate 1	Replicate 2	Replicate 3
Control	0	5	5	5
	12	20	15	25
	24	80	75	101
	48	220	350	450
0.1 mg/l	0	5	5	5
	12	10	15	20
	24	50	55	40
	48	100	250	250
1 mg/l	0	5	5	5
	12	8	7	12
	24	30	35	20
	48	90	100	150
5 mg/l	0	5	5	5
	12	5	7	12
	24	20	25	20
	48	70	80	70
10 mg/l	0	5	5	5
	12	5	7	4
	24	10	8	7
	48	30	30	40

When you choose *New* from the file menu Simply Probits opens a form in which you specify the initial structure of your experiment. To set up a growth experiment data set first click on the *growth analysis* radio button in the *Type of analysis* panel. If the cultures are sampled at regular time steps eg (12, 24, 48 hours) then check that the *Regular time periods* box is ticked and enter the time step. The number of time steps must also be entered. If the time steps are not regular then these must be entered in the lower left-hand panel. Now specify the number of replicates and the number of test concentrations used. Note that the number of control replicates can be different from that used for the test concentrations. Finally, type in the lower right hand box the actual concentrations used.

Now click on Ok and the data grid will open with the experimental design you have specified laid out ready for you to enter the experimental results

Rapid repeat use of the same experimental protocol

Most laboratories tend to use the same experimental protocol repeatedly. To speed up data entry you can save a design protocol from the setup form for future use. Click on *Save template* button and give the experimental protocol used a name and it will be saved. To use this protocol again simply select it by double clicking on its name in the list of templates in the upper right-hand corner of the setup form.

4. Instructions for use

Start **Simply Probits** in the normal Windows fashion either by clicking on the program icon or from the start button. If you prefer you can simply run the executable file.

Along the top bar of the program are three pull-down menus. These work in a similar manner to menus found in other common Windows programs.

- **File**: This pull-down menu is used to open and save a data file or to create a new dataset. Choose exit to close down the program.

- **Help**: For access to the help system. Context specific help for a particular window is obtained by pressing the right hand mouse button while the window is active.

- **About:** Information about the program version, other products we offer and PISCES Conservation Ltd.

Once either an existing data file has been opened or a new data set chosen from the File menu a workbook with a variable number of sheets in it will appear. The first sheet is a data grid into which you can either open existing or type new data. The second sheet contains predictions and intermediate calculations. The third and later sheets present graphs linked to the chosen growth curve including the estimated parameters.

The structure of your data

Data files are stored as CSV format files and are given the default extension .tox. These files can be opened and viewed using any word processor or text editing program and can also be imported into and from spreadsheets such as Excel. We advise you to create the files using **Simply Probits,** as this will ensure the correct data structure is produced.

Researchers with large amounts of data held in other formats that require them to be passed to Simply Probits should contact Pisces Conservation.

Opening an existing data set

To open an existing file select File | Open and choose a file. The program is supplied with two example files, Growth example.tox and Copepod example.tox, which will be installed in the same directory as the executable program. Growth example.tox is a typical data set produced by a growth inhibition study. Copepod example.tox is an example of data produced by an experiment to estimate the 48 hr LD50 of marine copepods.

Creating and editing a dataset

Choose *New* from the File dropdown menu to create a new data file. A setup window will appear in which you can define the structure of your data set. The different fields in this important window are described below

The radio buttons in the top left panel are used to select the type of analysis. Choose *Growth analysis* when the experiment is designed to compare the growth of a series of cultures exposed to the test conditions. Choose *Lethal dose calculation* if the experiment is designed to calculate LD or EC values.

At the top right is a list of the standard experimental templates that can be selected. A template defines the number of controls, the number and type of test conditions and the number of replicates. You can create your own templates by choosing a data structure and then clicking on the *Save as Template* button.

If a growth analysis is selected then the time steps at which the culture densities are measured can be selected. If the time steps are evenly spaced through time *select Regular time steps* and fill in the size of the time step and select the unit of time. If the time steps are not of identical magnitude then the time of each reading must be entered in the panel. Enter the times at which the cultures were counted starting at 0 (zero) (e.g. 0,24,48,72,96 hours). Press return after each entry. The number of control cultures and the number of replicate

cultures at each test concentration should also be entered. Note that it is assumed that the number of replicates at each test concentration is the same. If this is not so, as could happen if a culture is accidentally lost then this can be entered as missing data later. The number of test concentrations that were run is entered in the box labelled *Number of Test Concentrations* and the actual test concentrations typed in the lower right hand box with one number on each line.

If a lethal dose type calculation is selected then a different series of inputs must be chosen. Select *Same starting levels* if the initial number of organisms in each experimental culture is the same and type in this number in the box labelled *Initial Number*. The other fields are the same as those for a growth experiment described above.

Remember, if you are working with a standard experimental design you should make a template to speed up the initial setup procedure.

Having chosen the experimental parameters click OK and the program will open the data grid organised as defined by setup or a predefined template. Simply type in the experimental observations. Missing observations are marked with a *.

Seeing the results

Once all the data has been added, select the various tabs to see the results of the calculations.

Saving your data

The data currently displayed in the data window can be saved to disk by selecting File | Save or File | Save as. This will open a standard Windows file dialog.

Maximum size of the data set

Simply Probits is set to handle a maximum of 100 concentration levels and 100 replicates of each. If this is too small please contact PISCES Conservation Ltd as versions for larger data sets can be supplied

Choosing a method

The program chooses the method of analysis (growth or lethal dose) automatically when a data set is selected. When a new analysis is to be undertaken the user first selects new from the File menu and then selects the method which will be applied to the data.

Choosing the time period for analysis in a growth study

The calculations for a growth analysis should be undertaken over a period when the controls are showing exponential growth. Choose the *control growth* tab to examine the graph of the change through time in control culture concentration. If growth is exponential a plot of log concentration against time will give a straight line. To log the axis click on the *graph options* button and choose log Y at the bottom left-hand corner of the chart preferences window. To choose the time at which exponential growth ends use the drop down box below the graph labelled *set time at end of exponential period*. A line will appear on the graph marking the end of the time period over which calculations will be undertaken.

Graphs and results

Select the graph and results tab in the lower left-hand corner to see the results for the chosen method applied to the selected data. The output available in the tabbed workbook will depend on your data and the method chosen.

Growth analysis results

For a growth inhibition study the following tabbed sheets are present.

Data. This presents in a grid the data collected from the test.

Results. This sheet shows the results of the calculations arranged in columns. The information held in the columns is as follows:

Column 1. – Conc.-The concentration of the test substance in the culture. Controls are labelled as such.

Column 2 – Area -The area under the growth curve (see below for method of calculation)

Column 3 – Mean Area – The mean of the area estimates at each concentration level.

Column 4 - % inhibition – The mean area at each test concentration expressed as a percentage of the control.

Column 5. – Growth rate – The estimated instantaneous growth rate for each culture (see below for method of calculation).

Column 6 – Mean rate – The mean of the growth rates at each test concentration.

Column 7 - % rate inhibition – The difference in mean growth rate at each test concentration expressed as a percentage of the control growth rate.

Below the grid of intermediate calculations is a panel giving the EC10 and 50 calculations based on both the area calculations and the growth rate. With these calculations is given the estimated standard error of the log EC value. The upper and lower 95% fiducial limits can be estimated as the calculated LC value ± the antilog base 10 of 1.96 times the standard error value.

Control graph. This sheet shows the average growth through time of the control cultures. It is used to determine the period over which exponential growth is observed. When first selected an untransformed plot of concentration (number of organisms) against time is presented. If growth is exponential then a plot of log concentration against time will give a straight line. To log the data click on the graph options button situated below the graph and select log Y. Other options can also be chosen to change the look of your graph. Also below the graph there are buttons that can be used to print, save or export the graph.

Biomass integral. This sheet shows the change in the mean area under the growth curve expressed as a percentage of the control area for each test concentration. Below the graph there are buttons that can be used to change the plot and print, save or export the graph.

Growth rate. This sheet shows the change in the mean growth rate of the cultures expressed as a percentage of the control growth rate. Below the graph there are buttons that can be used to change the plot and print, save or export the graph.

Lethal dose analysis output

For a lethal dose calculation the standard probit analysis is used and the following tabbed sheets are present.

Data. This presents in a grid the data collected from the test.

Probit graph. This sheet presents the graph of the 'empirical probits'. Immediately below the graph there are buttons that can be used to change the plot and print, save or export the graph. Below the graph there is also a panel which gives the results of the probit analysis. These comprise:

1. The LC or EC value for the percentage level chosen by the user using the LC/EC drop-down list.

2. The standard error of the estimated log LC value.

3. The upper and lower 95% fiducial limits. These are calculated as LC value ± the antilog base 10 of 1.96 times the standard error value

4. The Chi-squared value, which is the weighted sum of squares of the discrepancies between the expected and observed numbers, killed. The expected value is the degrees of freedom and a significantly large value indicates that the variance has been under estimated by a factor Chi-squared/(n-2), where n is the number of dosages tested.

5. The g statistic of Finney. A small g value indicates that it is appropriate to estimate the

fiducial limits as 1.96 times the standard error.

Outputting and exporting your results

You can print the graphs using the *print graph* button found beneath the graphs. This displays a preview of the graph that allows you to set margins etc. To export the graph into other programs press *Export Graph* button. For the best results when possible use the enhanced metafile format.

To print, copy to the clipboard or save your results and calculations use the appropriately labelled button. You will then be presented with a screen allowing you to choose what you wish to export or print. The copy and export results buttons use the data found below the graph (i.e. estimates LC 50 etc) plus where appropriate intermediate calculations found on the calculations grid (i.e. the mean area under each curve or the growth rates of the individual cultures) or both. The data is saved to disk in rich text format (rtf). A printout of the results is produced by clicking on *the Print results* button.

5. Methods of calculation

Growth analysis.

The procedures and calculations undertaken are as defined in ISO 10253. **Simply Probits** undertakes the following steps.

1. The growth of the control cultures through time is plotted and can be viewed by selecting the control graph tab. Calculations for the growth rate (see below) must be undertaken for the time period over which growth is exponential. This is the part of the growth curve that is linear if log densities are plotted. To plot log density click on the graph options button below the plot and select the log Y box. The time period over which growth is exponential is selected using the set exponential period drop down list on the Control Growth sheet. If no value is selected the program will default to the assumption that growth was exponential for the entire period of the experiment.

2. **Simply probits** then calculates the area under the growth curves using the equation:

$$A = \frac{t_1(N_1 - N_0)}{2} + \frac{(t_2 - t_1)(N_1 + N_2 - 2N_0)}{2} + .. + \frac{(t_n - t_{n-1})(N_{n-1} + N_n - 2N_0)}{2}$$

where

t_1 is the time in hours between the first measurement and the beginning of the test,
t_2 is the time from the second measurement and the beginning of the test,
N_0 is the nominal (initial) culture density and
N_n is the cell density at t_n .

The results for these calculations are given in column 2 of the results grid which is displayed by clicking on the results tab.

The mean values of A at each test concentration and for the controls is then calculated and placed in column 3 of the results grid. These mean values are then used to calculate the percentage inhibition using the equation:

$$I_{Ai} = \frac{\overline{A_c} - \overline{A_i}}{\overline{A_c}} . 100$$

where

I_{Ai} is the percentage inhibition for the test concentration i,
A_i is the mean area for test concentration i and
A_c is the mean area for the control.

The percentage inhibition calculations are given in column 4 of the results grid and a plot of the % inhibition against the test concentration is displayed by clicking on the integral growth tab.

3. Simply probit then calculates the growth rate, μ, per unit time for each test culture using the equation:

$$\mu = \frac{\ln N_L - \ln N_0}{t_L}$$

where

t_L is the time from the beginning of the experiment to the last measurement when growth in the controls was still exponential,
N_0 is the initial (nominal) culture density and
N_L is the culture density at time t_L.

The calculated growth rates are given in column 5 of the results grid and the mean growth rate for each concentration and the control in column 6. The percentage inhibition is calculated from these mean values using the formula:

$$I_{\mu i} = \frac{\overline{\mu_c} - \overline{\mu_i}}{\mu_c}.100$$

where c refers to the control and i to the test concentration.

4. Finally, the EC10 and EC50 values are calculated using the tabulated values for I_{Ai} and I_{ui} and displayed in the panel at the foot of the results grid. The estimates are obtained using the weighted regression method of Finney (1966).

A Standard probit analysis for Lethal Dose calculations

Probit analysis is undertaken using the maximum likelihood method of Finney (1966). The basic steps in the calculation are as follows:

1. The logarithm of the test concentrations is found.

2. The percentage of the test organisms affected (killed) is calculated for each test condition (chemical concentration). These percentages are then adjusted to take account of any losses experienced by the controls.

3. An empirical probit is then found by the program from an internal lookup table.

4. The plot of the empirical probit against concentration is then undertaken and a provisional line fitted to the graph by simple regression. This plot is shown on the tabbed sheet called probit plot.

5. Using this provisional relationship, expected probits are then calculated and used in conjunction with an internal lookup table to calculate a working probit.

6. A weighted regression is then undertaken using the working probits and the log of the test concentrations. The regression equation is then used to find the concentration for the desired level (eg 10, or 50%) of effect.

7. The variance and 95% fiducial limits are then calculated. The fiducial limits are calculated as 1.96 x the standard error. If the value of g is small then the fiducial limits presented can be viewed as reliable.

8. A Chi-squared statistic is also calculated. If this value is not significant with the number of terms used for the regression-2 degrees of freedom then there is no significant heterogeneity in the data.

6. Example applications

Simply Probits has been tested against a series of real data sets and the results calculated by other workers, usually working by hand and with graphs fitted by eye, compared with the output from *Simply Probits*. Here we give two sample data sets.

Growth analysis

The test data were as follows:

Concentration	Time	Replicate 1	Replicate 2	Replicate 3
Control	0	5	5	5
	12	80	55	75
	24	180	175	201
	48	320	350	450
0.1 mg/l	0	5	5	5
	12	75	55	60
	24	150	165	170
	48	295	320	415
1 mg/l	0	5	5	5
	12	40	45	50
	24	95	105	95
	48	180	195	170
5 mg/l	0	5	5	5
	12	5	7	12
	24	20	25	20
	48	70	80	70
10 mg/l	0	5	5	5
	12	5	7	4
	24	10	8	7
	48	30	30	40

Giving the following results

File name = Growth Example.tox   Date = 19/10/98

Biomass Intergral
EC10 = 0.1164 Estimated log SE = 0.4364   Number Obs. = 4
EC50 = 0.8522 Estimated log SE = 0.2479   Number Obs. = 4
Growth Rate
EC10 = 0.5482 Estimated log SE = 0.5379   Number Obs. = 4
EC50 = 8.189   Estimated log SE = 0.3721   Number Obs = 4

Intermediate calculations were as follows.

Conc.	Area	Mean Area	% Area Inhibition	Growth Rate	Mean Rate	% Rate Inhibition
Control	4890			0.08664		
Control	4710			0.08851		
Control	5862	5154		0.09375	0.08963	
0.1	4320			0.08495		
0.1	4410			0.08664		
0.1	5100	4610	10.55	0.09206	0.08788	1.952
1	2550			0.07466		
1	2820			0.07632		
1	2610	2660	48.39	0.07347	0.07482	16.53
5	570			0.05498		
5	714			0.05776		
5	558	614	88.09	0.05498	0.05591	37.63
10	210			0.03733		
10	210			0.03733		
10	222	214	95.85	0.04332	0.03933	56.12

These calculations have been checked by hand and found correct.

Lethal dose calculations

As a check on the accuracy of **Simply Probits** a detailed comparison was made with a fully described example calculation from Finney (1966)

The observed data and intermediate calculations was as follows:

Conc.	Log conc.	No experimental animals	No alive at end	Percentage killed	Percentage adjusted for control deaths	Empirical probit	Expected probit
0	-	48	47	2	0	-	-
65	1.81	47	43	9	7	3.52	3.3
130	2.11	52	37	29	28	4.42	4.7
260	2.41	44	6	86	86	6.08	6.1
390	2.59	45	1	98	98	7.05	6.9
520	2.72	49	0	100	100	Inf.	7.5

and the final results calculated by Finney were :
LD50 = 156 mg/l
Standard error of log LD value = 0.026
Fiducial limits (95%) = 175 and 139 mg/l

Simply Probits using the same data gave the following intermediate results

Conc.	Log conc.	No experimental animals	No alive at end	Percentage killed	Percentage adjusted for control deaths	Empirical probit	Expected probit
0	-	48	47	2	0	-	-
65	1.81	47	43	9	7	3.52	3.3
130	2.11	52	37	29	28	4.42	4.7
260	2.41	44	6	86	86	6.09	6.1
390	2.59	45	1	98	98	7.05	6.9
520	2.72	49	0	100	100	Inf.	7.5

and final estimates as follows:
LD50 = 154.8 mg/l
Standard error of log LD value = 0.025
Fiducial limits (95%) = 173.1 and 138.4 mg/l

Simply Probits gave a slightly different result from Finney. However, a small difference is to be expected as Finney fitted a line by eye. It can be concluded that **Simply Probits** gave the same results as Finney.

7. References

Finney (1966) Probit Analysis. Chapman & Hall, London.