A simplified step-by-step guide to non-linear regression analysis of herbicide bioassay, by using a spreadsheet

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1   Introduction

Bioassays have been long regarded as the basic methodology in the screening assessment of biological activity and selectivity of herbicides, as well as in the evaluation of potential risks of side-effects into the environment (Streibig & Kudsk, 1993). From a methodological point of view, these kind of assays are traditionally organised by using several doses of one or more herbicides/preparations (often with replicates) and measuring the response of some selected test-plant species.

Following the principles established in ecotoxicological studies with other xenobiotic compounds, it is generally recognised that bioassay results should not be analysed by using Multiple Comparisons Procedures (MCP). Indeed, even though we include in the experiment some particular dosages, we are not interested in the response at those dosages in itself, but we rather aim at defining the overall shape of the entire dose-response relationship (see for example Cousens, 1988).

Starting from the seminal work of Finney (1978), the most appropriate approach to data analysis is recognised as follows: (1) fit some sort of sigmoidally shaped dose-response curve into the observed results; (2) verify the goodness of fit (i.e. verify that the curve provides a good description of the dataset); (3) derive from the fitted curve some meaningful estimates, to be used as descriptors of phytotoxicity and for comparison purposes. One fundamental thing, that is often neglected, is that such estimates should always be accompanied by some measure of uncertainty (Onofri *et al.*, 2010).

The most used descriptors of phytotoxicity and toxicity in general are the so-called Effective Dose levels (ED), i.e. the doses causing some particular level of damage on the test plant (fig. 1). Among these, the ED50 (dose required to reduce plant growth half-way between upper and lower asymptote) is the most widely known, though other ED levels have played some role in herbicide phytotoxicity studies, such as the ED10 (dose required to reduce plant growth 1/10 between upper and lower asymptote), which may be assumed as No Observable Effect Level (NOEL; Pestemer & Gúnther, 1993) and the ED90 (dose required to reduce plant growth 9/10 between upper and lower asymptote), which can be assumed as Minimum Lethal Herbicide Dose (MLHD; Haage *et al.*, 2002). Of course, other ED levels may be of interest in each experimental situations.

If two (or more) herbicides/formulations have to be compared, the relative potency or efficacy (R) may be also determined, as the ratio between similar ED levels (for example, R = $ED50_A$ / $ED50_B$). It has been shown that the comparison of doses giving the same effect ('horizontal assessment') may be used as an indication on how much the dose can be relatively re-
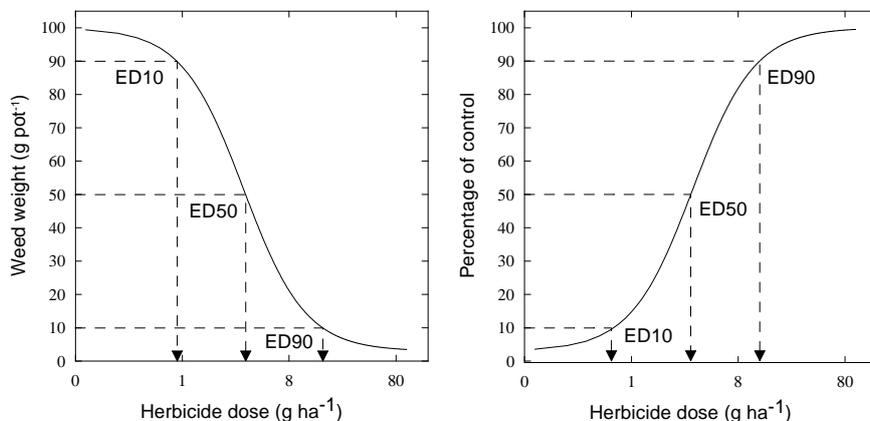
Figure 1. Examples of dose-response curves. The most important ED levels are outlined

Figure 1: Example of a dose-response curve. The most important
ED-levels are outlined (ED10, ED50 and ED90).

duced/increased for herbicide B to obtain the same effect of herbicide A.
Such a decrease/increase is significant whenever R is significantly different
from 1. The same approach can be used to compare the phytotoxicity of the
same herbicide when used in different pedo-climatic or floristic conditions. It
should be pointed out that, unless the dose-response curves are parallel (Fig.
2, left), R is specific to the selected ED level (2, right; Ritz *et al.*, 2006).

To ensure that the estimated ED or R values are not biased, an appropri-
ate statistical procedure has to be followed, considering all the peculiarities
of herbicide bioassays, with respect to bioassays with other xenobiotics. In
particular, it should be considered that: (1) responses can be either quanti-
tative (for example, the weight of weeds surviving the treatment) or quantal
(for example, the proportion of surviving weeds); (2) lower asymptote (re-
sponse at very high doses) may frequently be different from zero; (3) in some
cases response curves may not be symmetric along the x-axis; (4) growth
stimulation at low doses (hormesis) may occur with some herbicide families.

Rational techniques for analysing herbicide bioassays have been thor-
oughly revised and developed by Streibig *et al.* (1993a,b). More recently,
Ritz (2010) has made the attempt of providing a unified approach to dose-
response modelling. However, we feel that selecting an appropriate working
procedure may still be a complex task, especially for those who are not very
experienced on the statistical matter. Therefore, we made the attempt of
producing a simplified step-by-step framework, to enable people at all levels

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of statistical proficiency to reach unbiased estimates of ED-levels and relative efficiencies. The proposed procedure may be as follows:

- preliminary actions;

- selection of the dose-response model;

- selection of correct starting values for parameters;

- assessment of model adequacy;

- possible adoption of stabilising transformations;

- possible redefinition of a simplified model;

- estimation of EDs and R with standard errors.

We are aware that such an approach may sound somewhat too dogmatic, thus we would like to state clearly that there are other correct ways to appropriately analyse the results of herbicide bioassays.

2 A possible framework

2.1 Step one: preliminary actions

Following the advice in Ritz (2010), we would recommend avoiding any type of data pre-processing, such as a logarithmic transformation of doses and/or the normalisation of responses (for example relative to the untreated control). Indeed, the logarithmic transformation of doses, though necessary to set an approximately symmetric sigmoidal response pattern, should be tackled by selecting an appropriate model (see later). On the other hand, the normalisation of responses is only recommended with quantal data, that may be succesfully transformed into proportions. In this case, the Abbot's formula may be considered to account for baseline mortality on the untreated control (Abbott, 1925).

Step two: select a dose-response model

Among the possible choices, we recommend to stick to the traditional log-logistic equation:

$$Y = C + \frac{D - C}{1 + exp(b(log(dose) + log(a)))} \tag{1}$$

where Y is the expected response, C is the lower asymptote (the response at very high herbicide doses), D is the upper asymptote (response on the untreated control), b is the slope of the curve around the inflection point, while a is the dose giving a response half-way between C and D.

This model would probably fit in most of the cases, while a Weibull model (i.e. a Gompertz equation based on the logarithm of dose) should be selected in case of clearly asymmetric responses:

$$Y = C + (D - C) \cdot exp(-exp(b(log(dose) + log(a)))) \qquad (2)$$

The above models, will work with both decreasing curves (for example based on growth of test-species, that is expected to decrease as dose increases) or increasing curves (for example based on growth inhibition, that is expected to increase as dose increases), by changing the sign to the b parameter.

In case of hormesis (stimulation of responses at low herbicide doses), a peaked model may be suitable (Brain & Cousens, 1989):

$$Y = C + \frac{D - C + F \cdot dose}{1 + exp(b(log(dose) + log(a)))} \qquad (3)$$

even though the use of such an equation should be carefully evaluated, as its mathematical properties are not as good as those of the other models. When the interest does not lie directly on the stimulating range, good results may be obtained by removing from the dataset the stimulating dose and fitting the equations 1 or 2.

In all cases, constraints can be put on the lower and/or upper asymptotes, which might be necessary to reach convergence or to improve the estimates of the other parameters. In particular, these constraints might be needed:

- for biological reasons;

- whenever the lower asymptote is not significantly different from 0 or negative;

- dealing with curves based on percentage weed control, when the upper asymptote is higher than 100%;

- when asymptotes are measured or known without any experimental error.

In the case of multiple curve analyses, curves may be forced to assume the same lower and/or higher asymptotes, whenever this is biologically reasonable. Furthermore, curves may be forced to assume the same slope (parallel curves), but such a step should be statistically justified (see later).

2.2 Step three: providing starting values

As the above models are intrinsically nonlinear, the estimation of the parameters C, D, b and a (also, F, if required) is carried out by iterative procedures, that require reasonable starting values. Basically, in the case of decreasing curves the highest and the lowest observed response values can be used as the starting points respectively for D and C (this is reversed, in the case of increasing response curves). A starting value for the inflection point can be easily estimated from observed data, selecting a dose value which gave a response approximately half way between the higher and the lower asymptote. A value of 1 will be appropriate in most cases for the slope (b), and for the parameters describing stimulation (F), if needed.

Other more appropriate procedures have been suggested to obtain initial parameter estimates (Ritz & Streibig, 2008), which may turn out useful in some circumstances. In case of difficulties, we suggest also to try and fit the model, after constraining D and C respectively to the maximum and minimum observed values. The estimated b and a can be used as starting values for fitting the complete model.

2.3 Step four: testing model adequacy

This is a fundamental step, to ensure that the estimated ED-levels and relative efficiencies are unbiased. Among all the possible procedures, we would recommend a graphical inspections of (1) observed and predicted values, plotted against doses; (2) residuals plotted against predicted values. These will immediately reveal any problem with respectively (1) lack of fit and (2) heteroscedasticity. With this respect, we would like to point out that, to our experience, heteroscedasticity is the rule, not the exception, in bioassay work. On the other hand, non-normality of residuals is of less concern, as nonlinear regression is rather robust against this kind of deviation.

In case of lack of fit, you should go back to step two and select another model. A direct comparison among alternative models may be based on the residual sum of squares (the lower the best) for models with the same number of parameters, or on the 'extra sum of squares principle' for nested models. We will give examples later on.

In the case of variance heteroscedasticity, we recommend to go to the next step.

2.4 Step five: transformation of data

As we mentioned before, heteroscedasticity is of main concern with herbicide bioassay data and it generally shows up with some sort of proportionality among residuals and fitted values.

Heteroscedastic data always require a stabilising transformation. We recommend the use of the Box and Cox (1964) family:

$$W = \begin{cases} \frac{(Y^\lambda - 1)}{\lambda \bar{Y}^{\lambda-1}} & \text{if } \lambda \neq 0 \\ \bar{Y} \log Y & \text{if } \lambda = 0 \end{cases} \tag{4}$$

where W is the transformed variable, Y is the untransformed variable, λ is the transformation parameter and $\bar{Y}$ is the geometric mean of the observations.

By selecting a value for λ, several transformations can be applied to the dataset: a value of 1 implies no transformation, a value of 0.5 implies a square root transformation, while a value of 0 implies a logarithmic transformation. The most appropriate value may be simply selected by comparing the residual sum of squares of the different fits.

The use of stabilising transformations has been criticised, as the original variable looses its biological meaning. This problem can be overcame by using a Transform Both Sides technique. (Streibig *et al.*, 1993b).

2.5 Step six: redefinition of the model

One of the biggest risks in nonlinear regression is 'overfitting', i.e. including in the model a higher number of parameters than that justified by the data. This results in higher standard errors and biased parameter estimates. Following the Occam's razor principle, we should always seek for the simplest model, that accurately fits into the observed data. For example, a lower asymptote may not be justified, either because it is not significantly different from 0 or because it assumes biologically unrealistic values (negative). In this case it should be removed from the model.

Whenever we need to compare several dose-response curves and calculate a relative efficiency value, we should fit them simultaneously to the same dataset, introducing a quantitative variable coding for the different series of data. In this case, it may be useful to reduce the number of parameters and let the curve assume different a values, but same D, C and b (parallel curves). This is very handy, resulting in one single relative efficiency, that is constant and independent on the selected ED-level. Unfortunately, such a behaviour is not always biologically realistic and the hypothesis of parallelism should always be tested.

As the consequence of the above considerations, at the end of the fitting process it might be necessary to see whether the model should be redefined. Alternative formulations may be compared by using the extra-sum of squares principle. An example of calculation will be given later on.

2.6 Step seven: estimation of EDs and relative efficiencies

Nonlinear regression analysis provides D, C, bb and a values, together with standard errors. Once we are sure the model is adequate, we can produce EDs and relative efficiencies as combinations of the above parameters.

Considering a log-logistic model, the a parameter coincides with the ED50, while other ED levels may be estimated as follows:

$$ED_l = a \cdot exp\left[-\frac{\log\frac{100-l}{l}}{b}\right]$$

where l is the desired effect level (10 for ED10, 90 for ED90 and so on). A standard error for these quantities may be estimated via the so-called 'delta method' (Huet *et al.*, 2004).

Considering a Weibull model, the above formula becomes:

$$ED_l = a \cdot exp\left[\frac{\log\left(-\log\left(\frac{100-l}{100}\right)\right)}{b}\right]$$

It should be emphasised that these ED levels are relative to upper and lower asymptotes and do not necessarily refer to the attainable weed control level. For example, if we have a dose-response curve with higher asymptote equal to 90 g plant^{-1} and lower asymptote equal to 20 g plant^{-1}, the ED50 produces a response reduction half-way between higher and lower asymptotes, which corresponds to a weight of 55 g plant$^-$1 (i.e. (90-20)/2 + 20) and to an absolute weed control of 38.9% (i.e. (1 - 55/90) x 100). It is always necessary to keep in mind that the effect levels produced by EDs coincide with the absolute weed control levels only when the lower asymptote is equal to 0.

Therefore, if one is interested in the dose producing a certain absolute control level, he should seek for the corresponding relative effect level, by using the following equation:

$$l = 100 - \frac{Y_l - C}{D - C} \cdot 100$$

where l is the relative effect level, while Y_l is the expected response. In the above example, if we are interested in the dose that would produce 50% weed control, we set $Y_l = 45$ and obtain $l = 64.3$. Indeed, we should estimate the ED64.3 level.

Once EDs have been estimated, they can be used to calculate relative potences, as the ratios between corresponding ED levels. If response curves are not similar and have different asymptotes, this calculation should be done with some care. Indeed, comparing the ED90 for one curve having D=100 and C=0 and another one having D=100 and C=90 may not be biologically meaningful. Also in this case, standard errors for R can be calculated by using the 'delta method'.

3 Software consideration

The above framework may be successfully followed by using generic statistical programmes, even though they may either be expensive or be difficult to use or require some programming to comply with the peculiarities posed by herbicide bioassays. Some specific tools for herbicide bioassays have been developed, within statistic environments such as SAS (Seefeldt *et al.*, 1995) or R (Ritz & Streibig, 2005). This latter package is very complete and up-end, even though it still requires some computer skills that may not always be available in students and technicians.

An alternative approach is to use a spreadsheet programme, such as Microsoft Excel. This is one of the most commonly used software and most biologists are well experienced with it, at least at an elementary level. To enable users to perform correct nonlinear regression analysis within Microsoft Excel, a freeware VBA macro has been designed (Onofri, 2005), making use of the add-in macro SOLVER.XLA (or SOLVER.XLAM) , which needs to have been previously installed on the system.

This macro gives the reader all the necessary tools to follow the above procedure, including the adoption of the Box-Cox transformation, that is simply done by specifying a λ value. In the end, the user obtains a graphical analysis of residuals, parameter estimates, EDs and Rs values, together with standard errors.

The starting point for the analysis is a matrix of input data, meeting the following criteria:

- they have to be organised in a database, with observations in rows, and variables in columns. Basically, at least one dose column and one response column are needed; one further column variable (with herbi-

cide/preparation code) is required in the case of simultaneous fitting of several response curves. The first row is reserved for variable names;

- the response variable must be numeric and measured on a continuous scale (fresh weight, dry weight, herbicide efficacy on a percentage scale, etc..). Quantal responses should have been previously transformed into proportions.

The usage of BIOASSAY97 is very straightforward and it does not need any particular information. The readers are referred to the hosting web site for detailed information (http://www.unipg.it/~onofri/Bioassay97/Bioassay97.htm). The following examples have been analysed by using this macro.

4 Example 1: determination of the ED50 for one herbicide/weed species combination

The following dataset is taken from an experiment carried out at the Department of Integrated Pest Management, University of Aarhus, Denmark (Pannacci *et al.*, 2010a). Plants of *Tripleuspermum inodorum* were treated with a sulphonylurea herbicide (tribenuron-methyl) at increasing doses and the fresh weight of treated plants per pot was recorded 3 weeks after treatment (tab. 1).

We can proceed to nonlinear regression analysis, with no preliminary action and select a log-logistic model with four parameters (Eq. 1). Looking at the observed data, we can provide 115, 1, 1 and 20, as starting values respectively for D, b, a and C, which should be good enough to obtain quick convergence.

On the result page, we first look at the 'residuals vs fitted' plot, showing clear evidence of heteroscedasticity (the graph is in figure 3). In order to select an appropriate λ value for transformation, we can tentatively refit the model several times with different λ values (i.e. 0.5, 0.25, 0, -0.5, -1.0) and record the corresponding residual sum of squares (i.e. 2380.5, 2192.0, 2056.0, 1920.5 and 1979.5, respectively for the six selected λ values). Therefore, we can finally select -0.5, as the maximum likelihood value for λ.

The final results are displayed in figure 4. We do not observe any evident lack of fit or deviation from basic assumptions; however, we can see that observed and fitted values show a somewhat 'odd' scale. This is due to the Box-Cox transformation that alters the scale of the response, even though the parameters are obtained on the original scale, thanks to the Transform Both Sides approach.

Table 1: Response of *Tripleuspermum in-odorum* to increasing rates of tribenuron-methyl (dataset from Pannacci *et al.*, 2010a)

Dose (g a.i. ha^{-1})	Fresh weight (g pot $^{-1}$)
0	115.83
0	102.90
0	114.35
0.25	91.60
0.25	103.23
0.25	133.97
0.5	98.66
0.5	92.51
0.5	124.19
1	93.92
1	49.21
1	49.24
2	21.85
2	23.77
2	22.46

Before considering the estimated parameters and ED values, we need to observe that the estimated value for the lower asymptote is 18.7, with a very wide standard error (14.8) and confidence limits including 0. Indeed, we can erase this parameter and refit the model. In BIOASSAY97, this is done by selecting a log-logistic model with lower asymptote constrained and entering the value of 0 in the corresponding field of the initial guesses form.

This simplified model shows a residual sum of square of 1999.4 (12 d.f.), that is slightly higher than that of the complete model (1920.6, with 11 d.f.). We can formally compare the two models on the basis of the extra sum of squares principle:

$$F = \frac{\frac{1999.4 - 1920.6}{12 - 11}}{\frac{1920.6}{11}} = 0.45 \tag{5}$$

The above ratio is expected to follow a F distribution with 1 and 11 degrees of freedom, which corresponds to a probability p = 0.516. Indeed, we can accept the null hypothesis that the simplified model is not significantly

worse than the complete model and, following the Occam's razor principle, we prefer the former.

It may be worth to mention that the values for ED10, ED30 and ED50 from the 'simple' model are respectively 0.40 ($\pm$ 0.114), 0.73 ($\pm$ 0.144) and 1.07 ($\pm$ 0.13) grams of tribenuron-methyl per hectar and that standard errors are much lower than those obtained with the 'complete' model (compare with fig. 4).

5 Example 2: quantal data

This dataset is taken from an experiment carried out at the Department of Agroenvironmental and Crop Sciences (Pannacci *et al.*, 2010b), aimed at assessing the allelopathic potential of water extracts of sunflower. Several lots of 50 seeds of *Sinapis alba* were put in Petri dishes and moistened with water extracts at increasing concentrations (three replicated Petri dishes for each concentration). The final number of germinated/ungerminated seeds was recorded at seven days after the beginning of the assay.

Bioassay methods for quantal data traditionally differ from those for quantitative data, mainly because the distribution of errors is not normal and homoscedastic. However, the above framework may still be used, as long as the necessary care is taken about basic assumptions. In this case, the number of ungerminated seeds for each Petri dish was preliminarily transformed into a proportion (tab. 2), which depicts a monotonically increasing response curve.

Considering model selection, we note that almost no seeds germinated at the highest extract dosage and, therefore, there is no evidence that the higher asymptote should be different from 1. On the other hand, a certain proportion of seeds did not germinate on the untreated check, probably due to some kind of dormancy. Therefore, we can select a log-logistic model with three parameters, constraining the upper asymptote to 1.

The results are displayed on figure 5, wherein no problems with heteroscedasticity and lack of fit are evident. This check is particularly urgent, due to the quantal nature of this dataset and, to our experience, the above finding is rather infrequent, as a main proportion of quantal datasets would require some sort of stabilising transformation.

Table 2: Response of *Sinapis alba* to increasing concentrations of water extracts of sunflower (dataset from Pannacci *et al.*, 2010b)

Concentration of extracts (g d.m. 100 mL^{-1} water)	Proportion of ungerminated seeds
0	0.18
0	0.14
0	0.06
5	0.10
5	0.18
5	0.10
10	0.06
10	0.22
10	0.16
17.5	0.76
17.5	0.52
17.5	0.52
25	0.94
25	0.98
25	0.98

6 Example 3: determination of relative efficiency

This dataset was taken from the same source as previous example Pannacci *et al.* (2010b); water extracts of two sunflower varieties were compared in terms of phytotoxicity to the same test-plant (*Sinapis alba*). Several lots of 50 seeds of *S. alba* were put in Petri dishes and moistened with water extracts at increasing concentrations (three replicated Petri dishes for each concentration). Radicle length were determined seven days after treatments (tab. 3).

A preliminary evaluation of this dataset shows that plants grown at the highest rate of extracts did never germinate, thus their average radicle length was 0, with no variability around the mean. This situation is very likely to impact negatively on variance homogeneity and it is not easy to be corrected

Table 3: Response of *Sinapis alba* to water extracts of two sunflower varieties (Sanbro and Oleko). Dataset from Pannacci *et al.*, 2010b)

Concentration of extracts (g d.m. 100 mL^{-1} water)	Root length of *S. alba* (mm)	
	Sanbro	Oleko
0	77.0	75.4
0	85.2	67.8
0	80.2	80.2
5	66.8	40.6
5	70.2	59.4
5	73.8	90.2
10	51.6	35.2
10	47.4	18.8
10	43.2	21.0
17.5	11.2	3.8
17.5	11.4	1.8
17.5	16.6	1.4
25	0.0	0.0
25	0.0	0.0
25	0.0	0.0

by using the Box-Cox transformation. In this situation, the highest dosage can be removed from further analysis and the lower asymptote can be accordingly constrained to 0 for both varieties.

Other constraints do not seem to have any prior biological justification, thus we start with a six-parameters model (i.e. two higher asymptotes, two slopes and two inflection points). Some preliminary work shows that the model would be adequate, by using $\lambda = 0.25$.

This first fit brings us to a residual sum of squares of 935.58 with 18 degrees of freedom. Estimates of higher asymptotes are respectively 69.9 mm for Sanbro and 77.9 mm for Oleko, with overlapping confidence limits (i.e. 57.3 - 82.5 and 63.6 - 92.1). Therefore, before looking at the relative potences, we redefine a simpler (more parsimonious) model, with common asymptotes, which shows a residual sum of squares of 974.88 with 19 degrees of freedom. By using the extra sum of squares principle, we can see that:

$$F = \frac{\frac{974.88 - 935.58}{19 - 18}}{\frac{935.58}{18}} = 0.756 \qquad (6)$$

which corresponds to a probability level of 0.396. Indeed, the null hypothesis of no difference between the two higher asymptotes cannot be rejected.

We proceed in this stepwise fashion and try a third fit, by forcing the two curves to assume the same slope (parallel curves), which brings us to a residual sum of squares of 1025.86 with 20 degrees of freedom. Also in this case, we can easily see that this value is not significantly higher than 974.88. A further (and final) attempt can be made to see whether the two curves also share a common inflection a, which would imply that they are, in fact, the same curve. The residual sum of squares for this final fit is 3533.26 with 21 d.f., which is significantly higher than 1025.86. Indeed, the final hypothesis of equal curves should be rejected.

The results for the parallel curves fit are reported in figure 6; we can conclude that Sanbro extracts are less phytotoxic than those of Oleko, showing a relative efficiency of 0.68 ($\pm$ 0.039). As the curves are parallel, the relative efficiency is constant and independent on the selected effect level.

7 Conclusions

In the end, it is important to reinforce the idea that the statistical analysis of experimental results should never be seen as the mindless application of some set of recipes. In providing the above framework, we would never intend to promote such an approach!

Do not forget a critical evaluation of results and keep in mind that there is a number of alternative ways to do correct analyses; in particular, more advanced and elegant techniques exist to deal with counts, proportions and heteroscedasticity problems (see for example Onofri *et al.*, 2010). However, we feel that in the case of routine experiments (and bioassays are often so), it is important to provide some clear and simple guidance, together with appropriate software tools, to be used by students, technicians or whoever is not particularly involved with statistics.

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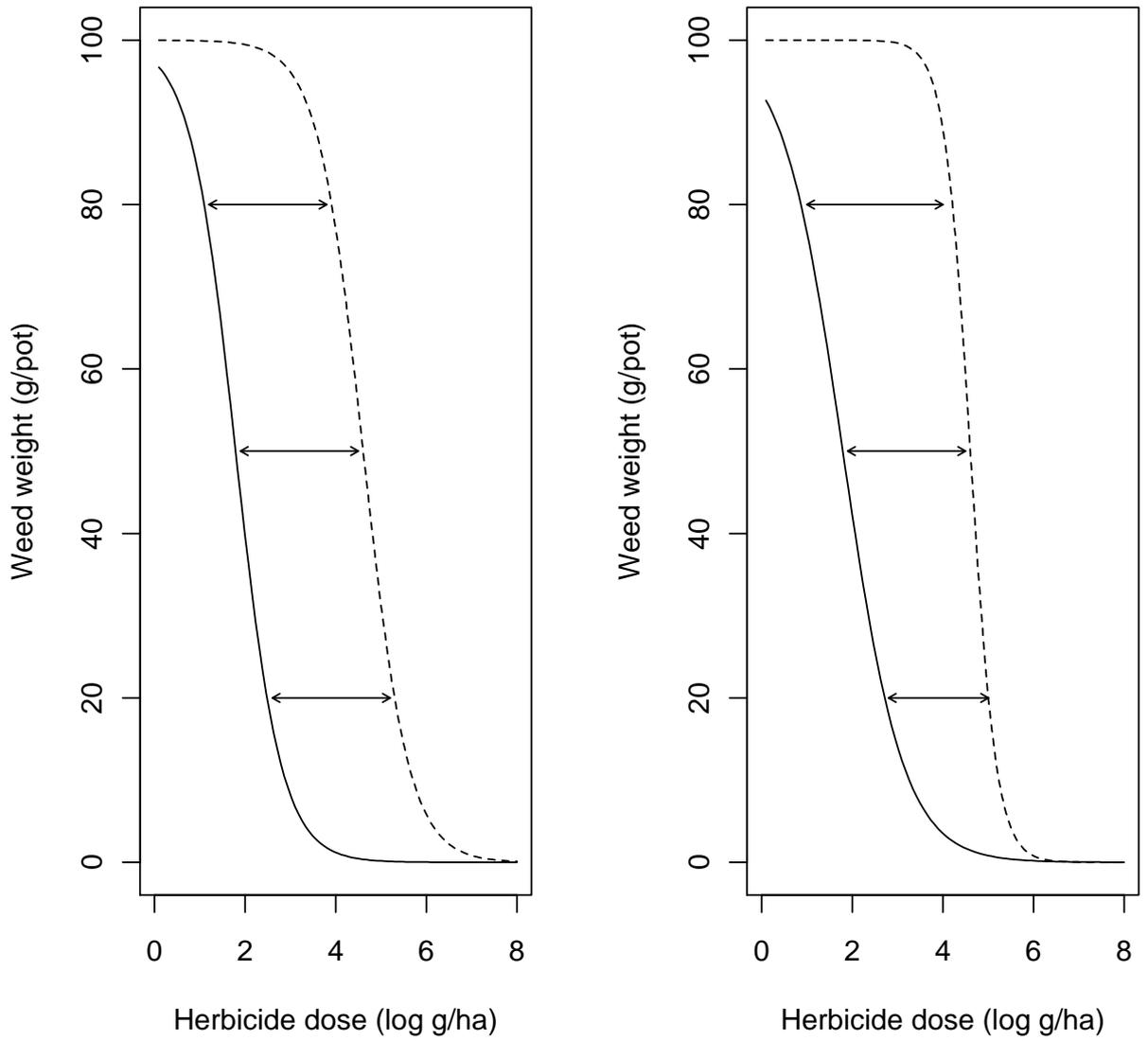
Figure 2: Horizontal comparison between parallel (left) and non-parallel (right) response curves. The arrows give a graphical representation of relative potencies.
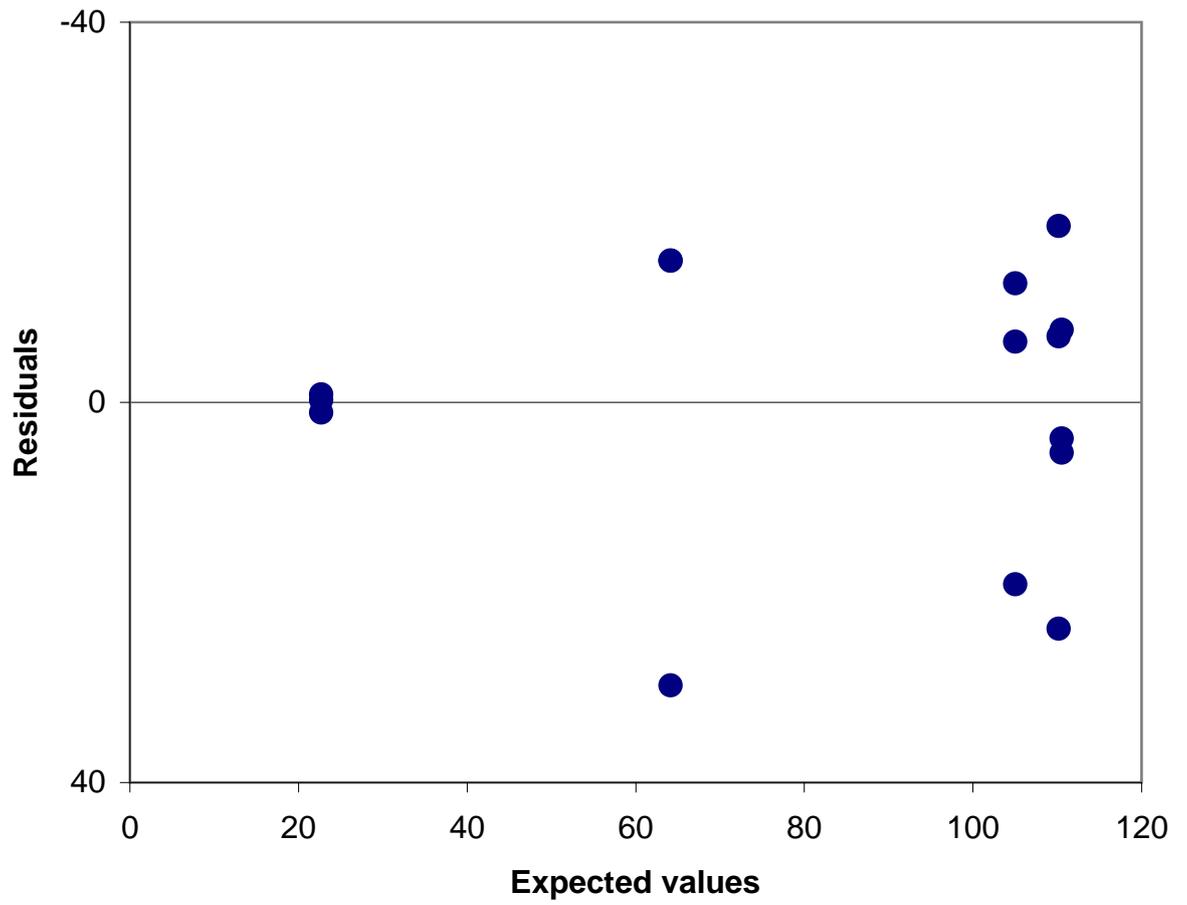
Figure 3: Plot of residuals against fitted values with heteroscedastic data.

Biological assay - non-linear regression analyses
DataFile: Dati_biosaggi.xls - Replicated trial / Response: FW - Dose: Dose
Fitted function: Log-logistic model (4 parameters)
Number of estimated parameters: 4
Lambda value for Box & Cox Transformation: -0.5 ; -2*log likelyhood: 7.56038508751863
Number of observations: 15
Residual Sum of Squares: 1920.58496428598
Degrees of freedom: 11

Parameter	Estimated value	St. err.	t value	Prob(t)	Upper limit (0.95)	Lower limit (0.95)
Higher asymptote	109.4017969	11.08701556	9.867560507	8.44518E-07	133.8041536	84.99944018
Lower asymptote	18.69457265	14.82765325	1.260791059	0.233470027	51.3300174	-13.9408721
Stimulation	0 not included					
Slope	4.169761281	4.85377862	0.858792333	0.408779493	14.85637589	-6.516853333
Inflection point	0.955038777	0.082346449	11.59781369	1.65014E-07	1.13628209	0.773795464
ED10	0.563861926	0.346083134				
ED30	0.779421031	0.191936204				
ED50	0.955038777	0.082346449				
ED70	1.170226398	0.301086966				
ED90	1.617592931	1.011643875				

Variance-covariance matrix

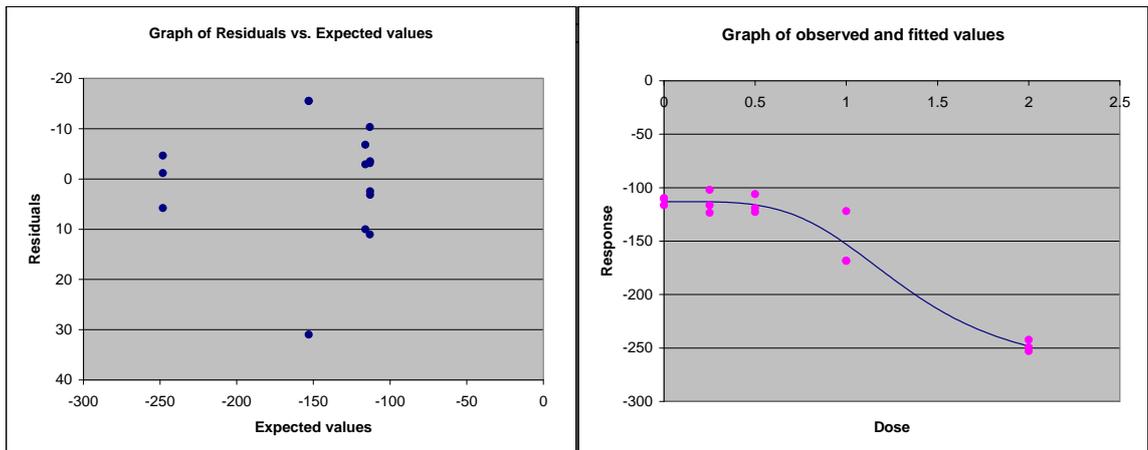| Higher as.	Slope	Inflection	Lower as.
122.921914	-32.35969019	-0.378486682	-94.21143048
-32.35969019	23.57469418	-0.027223493	71.31589668
-0.378486682	-0.027223493	0.006780938	-0.193858317
-94.21143048	71.31589668	-0.193858317	219.859301

Figure 4: Results of fitting the log-logistic model to the dataset in table 1, by using the Excel macro BIOASSAY97.

Biological assay - non-linear regression analyses
DataFile: Dati_biosaggi.xls - Quantal responses / Response: Proportion of - Dose: Extract concentration (g d.m. 100mL-1 water)
Fitted function: Log-logistic model with upper asymptote contrained (3 parameters)
Number of estimated parameters: 3
Lambda value for Box & Cox Transformation: 1 ; -2*log likelyhood: -2.74091642407509
Number of observations: 15
Residual Sum of Squares: 6.45112001786149E-02
Degrees of freedom: 12

Parameter	Estimated value	St. err.	t value	Prob(t)	Upper limit (0.95)	Lower limit (0.95)
Higher asymptote	1	Constrained				
Lower asymptote	0.130005401	0.025485675	5.101116653	0.000261318	0.185533917	0.074476886
Stimulation	0	not included				
Slope	-8.32124461	3.258948765	-2.553352388	0.025309164	-1.220605238	-15.42188398
Inflection point	17.15654614	0.468253282	36.63945729	1.09516E-13	18.1767824	16.13630988
ED10	13.17511479	1.561554742				
ED30	15.49560363	0.895680792				
ED50	17.15654614	0.468253282				
ED70	18.99552173	0.692562527				
ED90	22.34113934	2.099428194				

Variance-covariance matrix

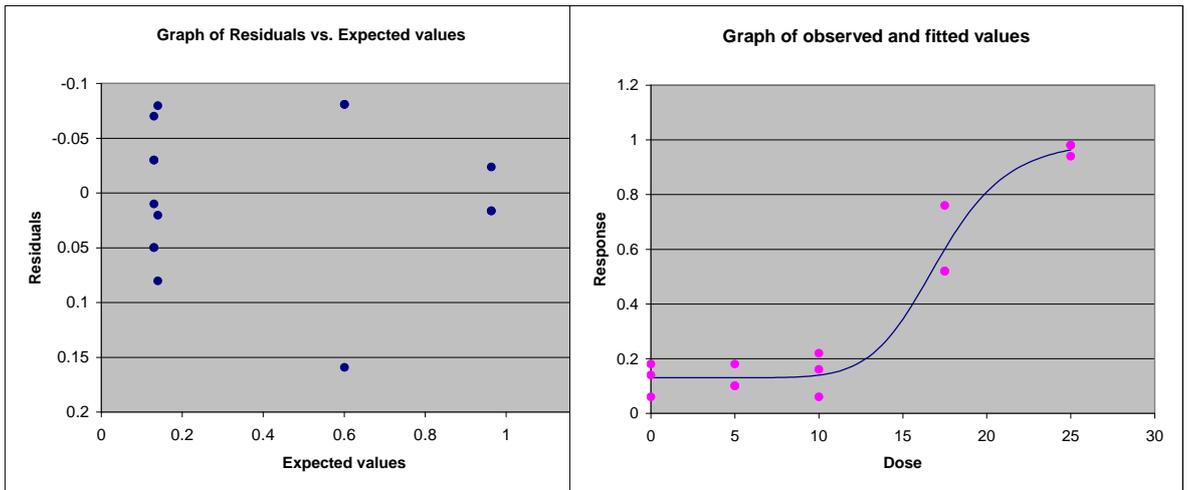| Slope	Inflection	Lower As.
10.62074705	-0.705180294	-0.0204942
-0.705180294	0.219261136	0.004245494
-0.0204942	0.004245494	0.00064952

Figure 5: Results of fitting the log-logistic model to the dataset in table 2, by using the Excel macro BIOASSAY97.

Biological assay - non-linear regression analyses
DataFile: Dati_biosaggi.xls - Varieties / Response: Length - Dose: Dose
Fitted function: Logistic
Number of estimated parameters: 4
Lambda value for Box & Cox Transformation: 0.25
Number of observations: 24
Residual Sum of Squares: 1025.85670509229
Degrees of freedom: 20

Parameter	Estimated value	St. err.	t value	Prob(t)	Upper limit (0.95)	Lower limit (0.95)
Higher asymptote - Curve 1	73.15663213	4.348055745	16.82513666	2.84505E-13	82.22651745	64.0867468
Higher asymptote - Curve 2	73.15663213	Same as Curve 1				
Slope - Curve 1	4.412230955	0.482544049	9.143685357	1.39434E-08	5.4188002	3.40566171
Slope - Curve 2	4.412230955	Same as Curve 1				
Inflection point - Curve 1	8.263726522	0.537276998	15.38075622	1.51443E-12	9.384466697	7.142986347
Inflection point - Curve 2	12.13850158	0.711024743	17.07184132	2.16528E-13	13.6216732	10.65532996
Lower asymptote - Curve 1	0	Constrained				
Lower asymptote - Curve 2	0	Same as Curve 1				
R (ED10) - Curve 2	0.680786378	0.038780057				
R (ED30) - Curve 2	0.680786378	0.038780057				
R (ED50) - Curve 2	0.680786378	0.038780057				
R (ED70) - Curve 2	0.680786378	0.038780057				
R (ED90) - Curve 2	0.680786378	0.038780057				

Variance covariance matrix			
18.90558876	-0.818874184	-1.307440666	-1.767301042
-0.818874184	0.232848759	0.2019058	0.2112369
-1.307440666	0.2019058	0.288666572	0.221353089
-1.767301042	0.2112369	0.221353089	0.505556185

| Dose	Expected	Observed	Residuals
0	152.5014276	153.6573432	1.155915579
0	152.5014276	149.629735	-2.87169262
0	152.5014276	156.0465155	3.545087887
5	148.6092941	131.6260934	-16.9832007
5	148.6092941	144.76284	-3.84645407
5	148.6092941	160.6986091	12.08931501
10	112.9790609	127.0124078	14.03334696
10	112.9790609	108.5800271	-4.39903378
10	112.9790609	111.6259857	-1.35307514
17.5	66.05983537	72.80419137	6.744355997
17.5	66.05983537	60.39884165	-5.66099372
17.5	66.05983537	56.7208189	-9.33901647
0	152.5014276	154.466094	1.96466637
0	152.5014276	158.4237839	5.922356261
0	152.5014276	156.0465155	3.545087887
5	151.7493375	149.0749248	-2.67441263
5	151.7493375	150.936669	-0.81266844
5	151.7493375	152.8356167	1.086279294
10	139.5729161	139.7567874	0.183871275
10	139.5729161	136.8217273	-2.7511888
10	139.5729161	133.6846121	-5.88830403
17.5	97.34559442	95.39266657	-1.95292786
17.5	97.34559442	95.81570429	-1.52989013
17.5	97.34559442	105.2537324	7.908138002
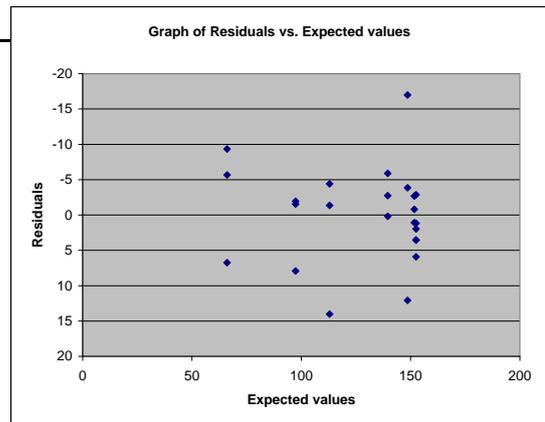
Figure 6: Results of a parallel-line assay with the dataset in table 3, by using the Excel macro BIOASSAY97.