MANUAL

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TOXICITY RESPONSE ANALYSIS AND TESTING

ToxRat® User Manual

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1 About this manual

Or: What to Expect

This manual was compiled to help you familiarise yourself as quickly as possible with ToxRat, so that you will be able to analyse your biotest data with ease, and can visualise the results in a pleasing format. In Chapter 2 you will learn about the most important details about ToxRat, i.e. the basic structure of ToxRat, and the principles of its functionality. We will also explain the meaning of the accompanying demo files and data templates, as well as the difference between manually and software-controlled analyses. It is important that you take the time to read these basic details!

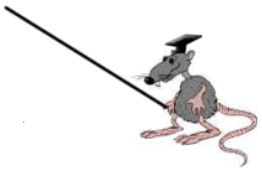
They will suffice if you don't like reading lengthy manuals and prefer getting started right away; you can use the accompanying demo files to get the hang of it, and to "learn by doing". You will find matching sections for specific questions on individual topics in the index. However, if you continue reading:

Detailed instructions are available as of Chapter 3. The manual follows the practical procedure for evaluating bio test data. We show you the step-by-step instructions, starting with the data input, selection of method and the presentation of the results, up to the creation of a report.

With the help of the integrated expert system and the use of the supplied templates, non-statistics experts can also run accurate statistical evaluations with ToxRat. Nonetheless, statistical terminology is used in the user interface as well as within this manual. Some additional tutoring in statistics is available under certain circumstances, however, this is limited to a minimum, since it would otherwise go outside of the scope of this manual. If necessary, please consult a corresponding statistics book.

Please note: Depending of exposure type, effect tresholds can be based on doses, rates, concentrations or levels. This can be defined in ToxRat. However, to simplify matters, in this manual, generally the terms NOEC and ECx are used.

At this point, we would like to introduce you to Emily:



Emily draws your attention to important text within this manual.

If Emily and text are in red, this information is very important. Always read these sections carefully!!

Emily in green will point out helpful information and give you tips on the current topic.

Emily in blue will provide you with more in-depth information about what is happening to and with ToxRat – *these are the sections for users who want to learn more!!*

2 Introduction or: What you should know about ToxRat

ToxRat is customized for statistical evaluations of ecotoxicology. The integrated contents of numerous Biotest Guidelines as well as the available expert know-how in the background for statistics (“Which test should I use?”) make this program unique.
In order to use these properties, you must enter the data in the pre-configured templates within the program. These templates are the key to ToxRat!

Using these templates ensures that also with complex biotests, ToxRat generates the results required by the associated guideline automatically, including validity checks and relieving you of the responsibility to select the correct statistical methods.
A complete list of the available biotest templates can be found in the chapter 10.

All ToxRat programs offer the same package of statistical methods and procedures. All meet the requirements of GLP, contain a validation document, and run a comprehensive report of the results. The various ToxRat programs differ only by type and scope of the templates for data entry and therewith also offer different levels of convenience.

ToxRat Standard only contains templates for manual evaluation.

ToxRat Monitor contains templates for manual evaluation and biotests for environmental monitoring in accordance with DIN EN ISO (environmental samples, waste water analysis, and dilution tests).

ToxRat Professional contains the manual and ISO EN ISO templates as well as those for bio tests in accordance with OECD, IOBC and OPPTS.

ToxRat Professional XT contains the same templates as in the Professional version with the additional import module for data obtained through specific biotest machines.

The different versions of ToxRat are set apart by the type and scope of the templates included in the terms of delivery. In this manual, we refer in general to the ToxRat Professional version, because the statistical procedures, the menus and registration cards, and the basic use are the same for all of the ToxRat versions. In those cases, where information only applies to a specific version of ToxRat, we will point this out.

2.1 Evaluation Levels

Many ecotoxicological biotests are done in accordance to specific guidelines, such as per OECD or DIN EN ISO. This is the special focus of ToxRat. The software uses integrated biotest-specific templates. Naturally, all biotest data can also be evaluated independently from the biotest templates, in case the needed guideline does not exist yet or because it is not part of the ToxRat program you have purchased. In these cases, general templates are available (Generic Data Sets).
Correspondingly, ToxRat knows two different evaluation levels:

Automatic evaluations of complete biotests pursuant to the guidelines
- are carried out with the help of specific biotest templates. Within these templates - according to the requirements of the relevant test guideline – the variables, measurement times, ecotoxicological endpoints and statistical procedures are automatically set as defaults in the program. User changes are possible (within the framework of the guidelines). The complete test sequence including the calculation of the derived variables (yield, growth rate) and validity criteria is started by the user with one command.
The respective templates are included with the purchase of the program and can be identified by file name based on the guideline number and bio test name.

Manually run, non-specific evaluations
- are carried out with the help of simple templates that enable the evaluation of individual variables. All statistical evaluations are manually selected and started by the user. The user must decide, if the variables to be evaluated are quantal or metric, if the data has been replicated or not, and if numerous measurement intervals are required. There is no validation check and any derived quantities such as yields and growth rates are not automatically calculated.
Under the supplied templates with the file names of „Generic data set metric responses“ and „Generic data set quantal responses“ an appropriate template can be selected.

2.2 Templates: Masterbooks und Workbooks

The above-mentioned data entry templates are in a Microsoft MS Excel ™ [1] compatible format (xlt, xls) and included with the software as Masterbooks and Workbooks.
ToxRat basically only processes the MS Excel 2007 xlt and xls formats. When working with ToxRat templates using MS Excel (see Chapter 3.4 und 3.5), please verify that these formats are maintained (not xlsx!).
All templates can be filled in and edited directly in ToxRat, using an integrated tool for data entry, the Workbookdesigner. MS Excel is not required. In particular, all evaluations are only processed within ToxRat, without the use of MS Excel functions. This means, that ToxRat can be run on any computer with the Windows operating system, regardless if MS Excel is installed on it or which version.
The basic usage of Masterbooks and Workbooks will be discussed in Chapter 3.3. Here a short summary is given.

[1] In the following adressed as MS Excel

Every template has a cover page („General Notes“) and at least one data sheet („InputRawData“).
The cover page provides general information about the current project, the test substance, the test system, and if necessary the ecotoxicological end points (NOEC, ECx, LID...), the measurement interval(s), etc... If it's a Biotest Workbook, then the name of the guideline is noted and the essential information about the test system has been pre-set. The contents of the cover page will be applied in the results report of the statistical evaluation.
The data sheets help with the input of the experimental data. Their structure follows certain rules, dependent on the data type.

Masterbooks are empty templates in.xlt format, in which you enter your own experimental data. Masterbooks are used exclusively for data entry, and cannot be evaluated directly in ToxRat, rather, they must first be converted to a Workbook (done with the simple click of a button).

Workbooks are completed Masterbooks, saved in .xls format and ready for evaluation in ToxRat. This means that Workbooks are used for data entry as well as for the evaluation. Raw data in Workbooks can be overwritten and edited. After the evaluation, the statistical results in the form of additional data sheets are available in Workbooks (result tabs).

It is up to you if you prefer to enter your data into an empty Masterbooks, or if you wish to use the Workbooks with the overwritable data.

2.3 Statistical Evaluation

The procedure for the statistical evaluation depends mostly on which template is used. Details will be given in chapter 4.

If you have chosen a **Biotest Template**, you should start a complete evaluation by hitting the RUN key. With this, all mandatory statistical parameters from the test guidelines (ECx, NOEC...) for all provided ecotoxicological end points (measured variable and calculated variables) will be calculated and the required validity criteria will be verified. In every case, the built-in default settings will ensure that a correct evaluation is completed. Individual user settings are possible.

If you have selected a **Generic Template**, each individual statistical evaluation will have to be done manually through the menu. However, you may use some of the built-in default settings and expert processes also with Generic Templates.

The fundamental accuracy of the program calculation is confirmed by the provided validation document. The used mathematical algorithms are explained in the first part of the validation document and the results are compared to literature and to independent calculations in MS-MS Excel. In the second part of the validation document, sample results are provided as an example, which you can reproduce applying your individual hardware setup.

3 Getting started – Creating a File

You have collected your data and would now like to evaluate it. First, you must decide if biotest data has to be evaluated following a specific guideline. If yes, then there is no need for any further intermediate calculations – instead, select the corresponding biotest template, enter the raw data –and ToxRat will take care of the rest. If not, then you, not ToxRat, calculate the data that you wish to statistically evaluate (yields, growth rates...).

To become familiar with ToxRat, it is recommended to start with a demo file for data input – as it is done in this manual.

3.1 Menu and Buttons on the start-up screen

Start the program by clicking on the ToxRat logo on your desktop.

You will see this tab (ToxRat name will vary depending on your program variant):

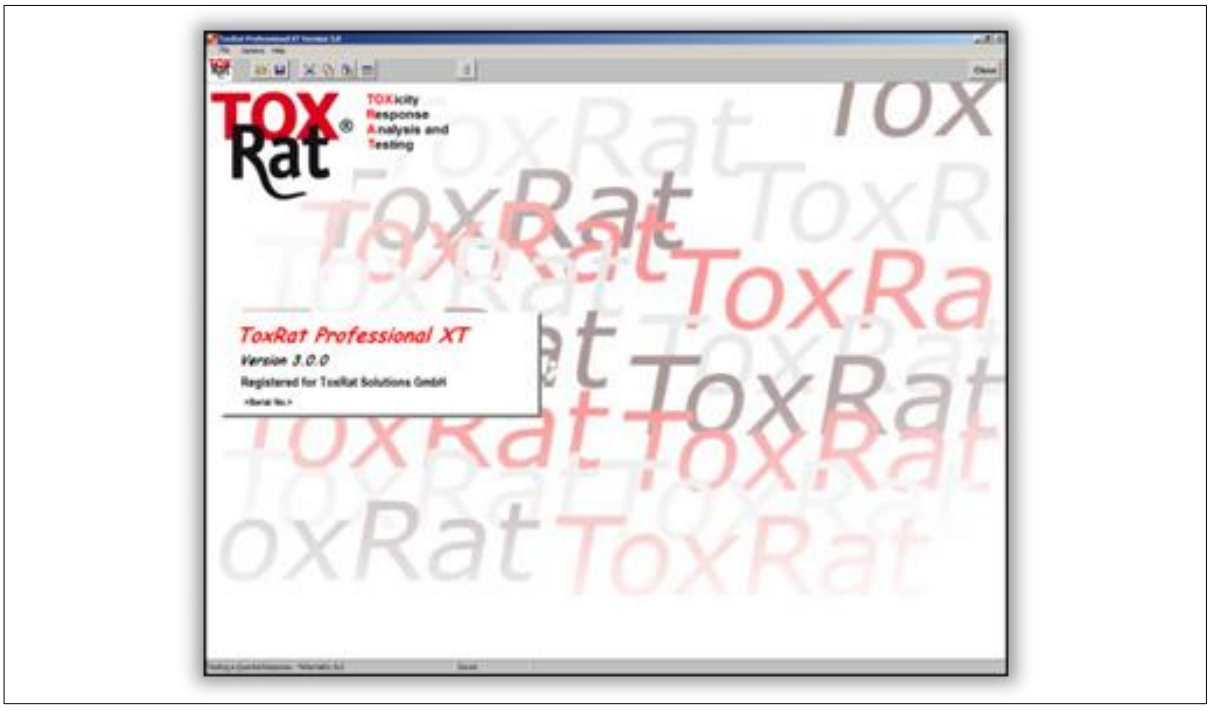


Figure 1: Start-up screen

The menu commands and buttons in ToxRat are context sensitive; This means that various commands and buttons will be available dependent on what you are doing at that moment. On the start-up screen you will find the following (Figure 2):

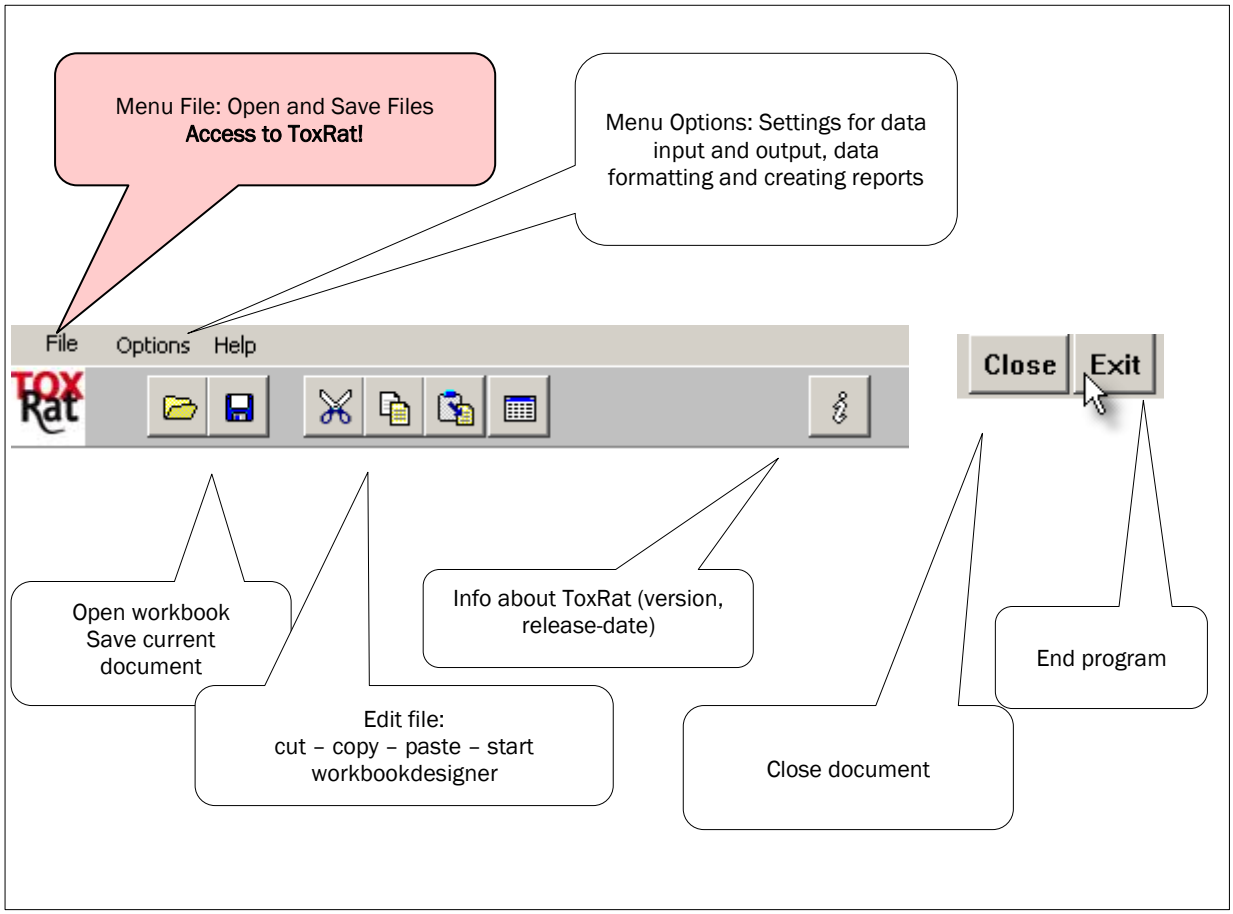


Figure 2: Menu items and buttons on the start-up screen

The menu items on the start-up screen are *File* and *Options*.

Under *Options* you will find a row of useful settings for data input and output, data formatting, and creating reports. Each of these items will be discussed in detail later. To begin, the default settings will suffice.

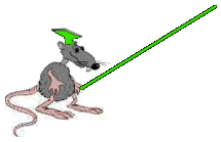
To start your first evaluation or to start one at all, you must first create and open a file. To do this, go to menu item *File*.

3.2 Workbook or Masterbook – That´s the question!

Remember: Masterbooks are empty templates. Workbooks already contain data, and are so to speak, pre-filled Masterbooks.

A quick and simple option to enter new data into ToxRat can be done by opening a corresponding Demo workbook (or an already available own workbook), where the existing data can be overwritten and saved under a new file name (*save as*). Unused cells can be left empty. The advantage with this option, is that it is easier to orient oneself with an already existing data structure.
If you would prefer to fill in an empty template, use one of the provided masterbooks instead. In this case, check the required data structure on the basis of a corresponding demo workbook!

In the remainder of this manual, we will refer to workbooks and demonstrate the next steps based on the demo workbooks included with this program. However, the following explanations also apply to masterbooks.



If you can't find a certain file in the opening dialogue box, please check that you haven't mixed up the commands „New Masterbook" and „Open File ". With „New Masterbook" only files with the extension xlt will be visible in the opening dialogue box; "open" shows only files with the extension "xls"!

All templates can be filled in and edited directly in ToxRat (integrated tools for data entry). MS Excel is not required. In particular, all evaluations are only processed within ToxRat, without the use of MS Excel functions. This means, that ToxRat can be run on any computer with the Windows operating system, regardless if MS Excel is installed on it or which version.

3.3 Opening a File

To introduce your data to ToxRat, please open a template using the file menu (Figure 3). Please note: There can be only one file open at a time. Once you open another file, the former file is closed. ToxRat will ask whether you want to save it using the current name. If you want to rename it, please use the "save as"- function instead.

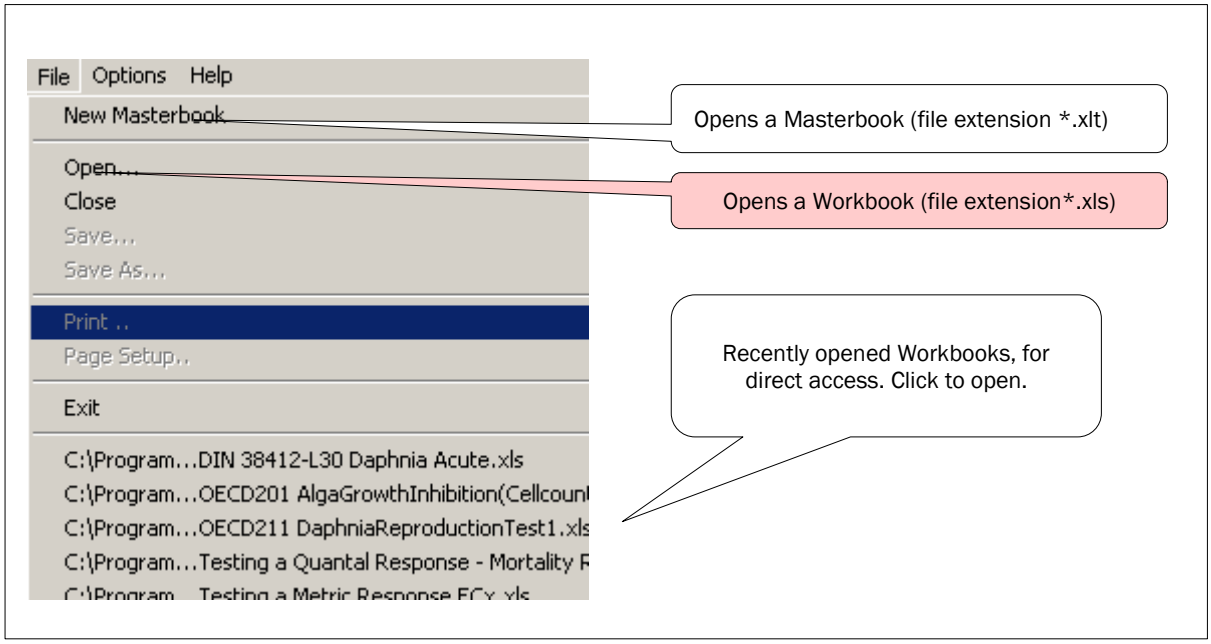
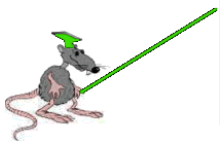


Figure 3: Menu „File" on the start-up sceen

Select menu items F*ile, Open*. In this dialogue box you will find all provided Demo Workbooks, sorted in various folders (If not: Activate the check box under Menu Items Options - Input/Output „Switch Workbookpath to Demopath").



If you wish to open a workbook from a different folder than the demo folder, (i.e. one of your own files) you can search for the file in the open dialogue box in the directory of your computer by clicking the small arrow in the search window.
The directory in which ToxRat searches first, can be changed under Options Input/Output. (Directories, Workbooks). The default setting is the provided demo directory.

In ToxRat Professional und ToxRat Professional ProXT you will now see the following window (Figure 4).
In ToxRat Monitor there are no directories OECD, IOBC und OPPTS, in ToxRat Standard there is not any Guideline-Directory, but you will only find the directories „Generic Data Sets Metric responses" and „Generic Data Sets Quantal Responses".

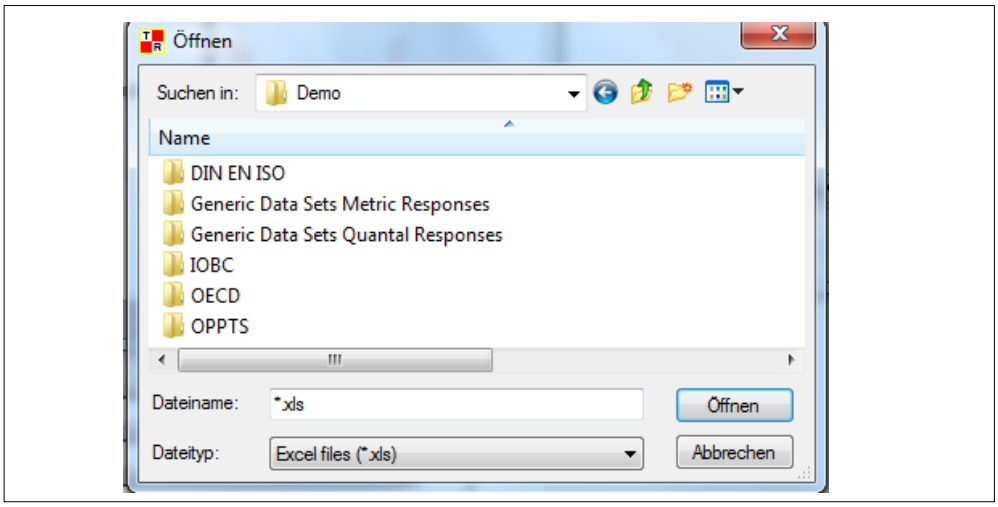


Figure 4: Dialogue box „File - open“

For ToxRat Professional and ToxRat Professional Pro XT users:
Select folder OECD, and then the subfolder “Substances“. You will now see a list of all available templates for the evaluation of bio tests as per OECD guidelines. Select Workbook *OECD 210 Fish Early Life Stage 2013.xls* and click on “Open”. This data record will be our example record in the manual.

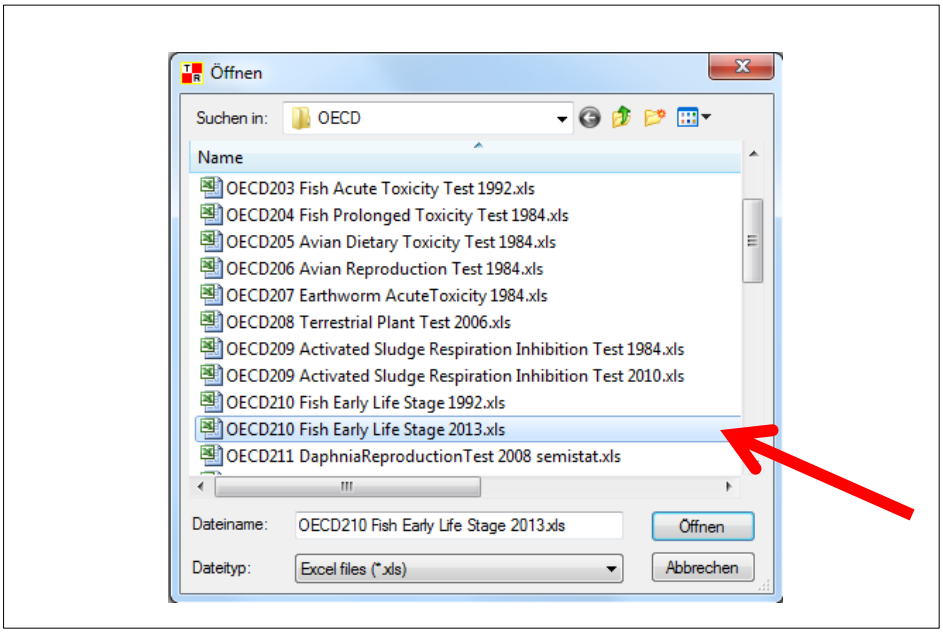


Figure 5: Dialogue box „File – open” – Directory Demo / OECD / Substances

Users of ToxRat Monitor, should now open Workbook DIN EN ISO 15088 2009 Acute Toxicity of Waste Water to Eggs of Danio rerio.xls from the DIN EN ISO / Water Quality Directory.Users of ToxRat Standard should now open Workbook Testing a Quantal Response 3 – Mortality replicated and at several Intervals.xls.
The following sections also apply correspondingly to these files. Any exceptions for ToxRat Standard and ToxRat Monitor will be pointed out.

3.3.1 The Structure of Workbooks und Masterbooks

Your screen should now look like Figure 6 :

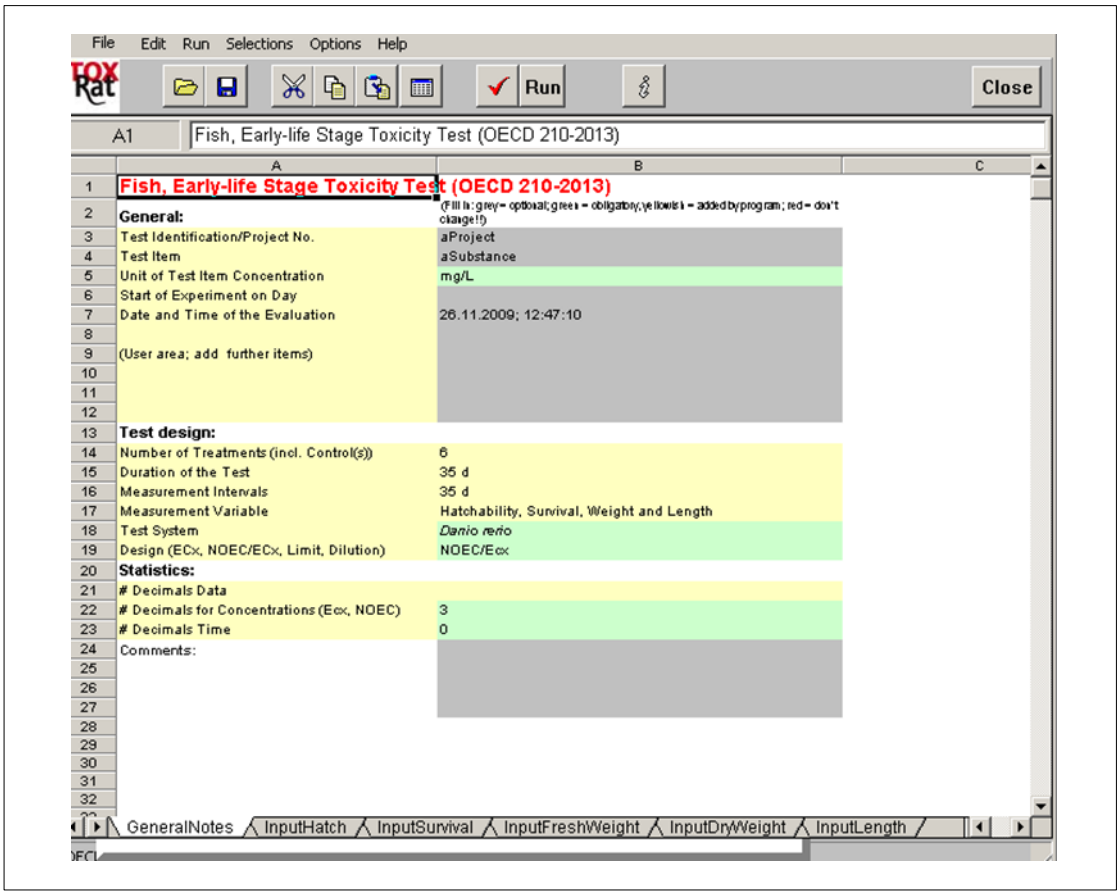


Figure 6: Cover page of a biotest workbook, called “General Notes”

Some further menu items and buttons, which are important for the evaluation, are now available – these will be explained in Chapter 4.1.

Let’s first take a look at the structure of the open workbook – it is similar than that of an MS Excel file.
Each Workbook has a cover page called „General Notes“ and one or more data sheets (Input-sheets). You can scroll back and forth through the individual pages by clicking on the tabs at the bottom of the screen.

Name, order, and the number of input sheets of a Workbooks must never be changed.

The colour coding is the same for all pages :

Fill in your data into the green cells. If no data are available, green cells can remain empty.

You may fill in any comments into the grey cells. They can also remain empty.

Yellow cells will be exclusively filled in by the program.

#Hatched Red text contains key formulas that run the program and therefore must never be changed.

3.3.2 The Cover Page (General Notes)

The General Notes contain general information about the current project, the test item, the test organism, the test design (number of treatments, test duration), and the measured variable(s). They furthermore determine which statistical end points are to be specified, and the number of decimal digits to be used for the presentation of the ECx and NOEC concentrations. Lines 3-19 of the cover page will be transferred over to the results and the report of the statistical evaluation.

The user must only fill in green and grey fields. No fields must be added or deleted in "General Notes"..

The **Biotest Workbooks** are sorted by test guideline (DIN EN ISO, OECD, IOBC, OPPTS). In cell A1 of the cover page the guideline is shown. Thereby, important information on the test system and and relevant data about the test system and the statistical evaluation are pre-set by default settings. Our examples:

ToxRat Professional: see Workbook OECD210, sheet General Notes
ToxRat Monitor: see Workbook *Din EN ISO 15088*, sheet General Notes.
ToxRat Standard: contains no Bio Test Workbooks, please continue with “Generic Data Workbooks”
The content of rows 14, 15, and 16 ("Number of Treatments", Duration of Test", "Measurement Intervals") is automatically updated by the software once data is read, following the preconfigured data structure of the worksheet – do not enter any information manually!

B5 Unit of Test Item Concentration: Enter unit of test substance concentration (e.g., µg/L, mg/L, etc.) In the case of a dilution test, this will be percent by volume (% vol/vol). This is optional, however, if this item is omitted all of the dose/concentrations mentioned in the results will not carry the unit specification

B17 Measurement Variable: This is a list of the variables that can be entered into the Workbook (these entries are not mandatory – some datasheets can remain completely empty as well). ToxRat will assign the variable type (quantal / metric) and therefore the selection of appropriate statistical methods for you. The content of this cell must not be altered.

B18 Test System The test organism to be used in accordance with the test guideline is preselected. If another species was used, this can be entered here (this is important if various validity criteria apply for different organisms, e.g. OECD 201 or OECD 206)

B19 Design The information in this cell governs the type and scope of the statistical evaluation carried out by ToxRat.

The default end points required in accordance with the guideline are preset. The preconfigured design will unsually not require any adjustments. If nevertheless required: ECx: ToxRat will calculate effect levels only. NOEC/ECx: ToxRat calculates threshold and effect levels, Limit: ToxRat carries out two-sample-tests, Dilution: ToxRat calculates the LID (Lowest Ineffective Dilution; only applicable for dilution tests in accordance with DIN EN ISO).

B22 and B23 Decimals Determines the number of decimal digits of ECx and NOEC concentrations, test concentrations and measurement times to be displayed (for details see chapter 6.4.2).

The „**Generic Data Workbooks**“ are available in two basic variants: Generic Data Sets Quantal Responses (for quantal variables) and Generic Data Sets Metric Resonses (for metric variables).

Quantal Variables are measured values that are recorded in the form of distinct categories or conditions. Their values are dimensionless. They can always be expressed as a percentage or a portion of a basic amount. Typical ecotoxicology examples are alive – dead (number of survivors from x introduced), hatched – not hatched (number of hatched from x introduced).
Metric Variables are measured values that can be measured with any accuracy, therefore they are of a continuous nature. They always have a certain dimension. Typical ecotoxicological examples are weights, lengths, metabolic rates.
Numbers, such as cell counts or number of offspring may be handled as metric variables, provided they are not limited to a maximum number. Therefore, offspring number in the Daphnia Reproduction Test (OECD 211) is metric, whereas the number of larvae hatched from a certain number of eggs introduced in Fish-Early-Life-Stage-Test (OECD 210) is quantal.

Demo file (all ToxRat variants):

Testing a Quantal response 3 – Mortality replicated and at several Intervals.xls
Testing a Metric response 4 at several Intervals.xls

In cell A1 of the cover page, it is noted whether the Workbook deals with quantal or metric variables. ToxRat applies different statistical methods depending on which type of generic workbook is used. The data entry structure also differs depending on the type of variable. Therefore, the accuracy of the calculation relies on the correct template the user chooses!

When using a Generic Data Workbooks it is **your responsibility** to characterize the correct type of variable to be evaluated and to select the appropriate Workbook (quantal or metric).

With a Generic Data Workbook, only one variable can ever be evaluated. For the cell meanings in the cover page, see Biotest workbooks, with the following exceptions:

B17	Measurement Variable:	Generic Data Quantal Responses: Possible variables are mortality, survival and emergence. Depending on the variable, certain key words in the data input sheets are required – these are pre-set if the correct template is selected (see file names). Generic Data Metric Responses: You can enter the name of any metric variable, e.g. “Weight” or “Length”. The name which is entered here, will be listed in the result tables („Result of the Probitanalysis with <variablenname>…”).
B18	Test System	The test organism is entered here. You can enter any organism, is not used by the program, only reported.
B19	Test Design	Not available, no input possible – Selection of the statistical end points is done manually.

3.3.3 The data sheets (Input Raw Data)

The data sheets of a Workbook allow the input of the actual test data. In Biotest Workbooks, the names and number of the data sheets are specific to the selected biotest, i.e. in an Algae Growth Inhibition Test in accordance with OECD 201, cell numbers (or extinction or fluorescence) are expected, in a Daphnia Reproduction Test (OECD 211), the input of the number of offspring is expected, while the Fish Early Life Stage Test (OECD 210) offers data sheets for hatching, survival, weight, length, etc. Simply use the various demo files to get an impression!

You can use the Biotest Workbooks for a simultaneous analysis of multiple variables as well as for different variable types (quantal and metric). ToxRat generates the quantal/metric assignment automatically, relieving you of the responsibility to select the correct statistical methods.
For the Generic Data workbooks only data for individual variables can be entered. The selection of this data and their description must follow certain rules. See above, Explanation for Cell B17.
Regardless of the workbook and variable type, the following structure of the data sheets in general applies:

The treatments (concentrations, doses, dilution levels...) are vertically arranged in columns and must be entered in ascending order. That is, in the first column is the control, followed by the solvent control (if existing), and then the test approaches in ascending concentration. This order of test approaches must absolutely be adhered to .

A Positive Control or External Standard can be optionally entered in the last column and is identified by the program by term such as "Positive Control" or "Standard" instead of a concentration or dilution. Per default, a positive control is excluded from statistical calculations. However, it can be included at users option, see Chapter 4.4.

Replications and measurement times are arranged horizontally, therefore in rows. If there are several measurement intervals, then the replications of a measurement interval are summarized in blocks.

Structure of a data sheet for quantal data

Our example files:
ToxRat Professional: Workbook OECD210, sheet Input Hatch
ToxRat Monitor: Workbook *Din EN ISO 15088*, sheet InputRawData. The number of introduced and the number of deaths are recorded. Important: Since this is a waste water test, the treatments are shown as dilution levels. For the determination of EC values, ToxRat converts these into volume-percent-parts (%Vol/Vol).
ToxRat Standard: Workbook *Testing a Quantal response 3 – Mortality replicated and at several Intervals.xls,* sheet InputRawData. The number of introduced and the number of deaths are recorded.

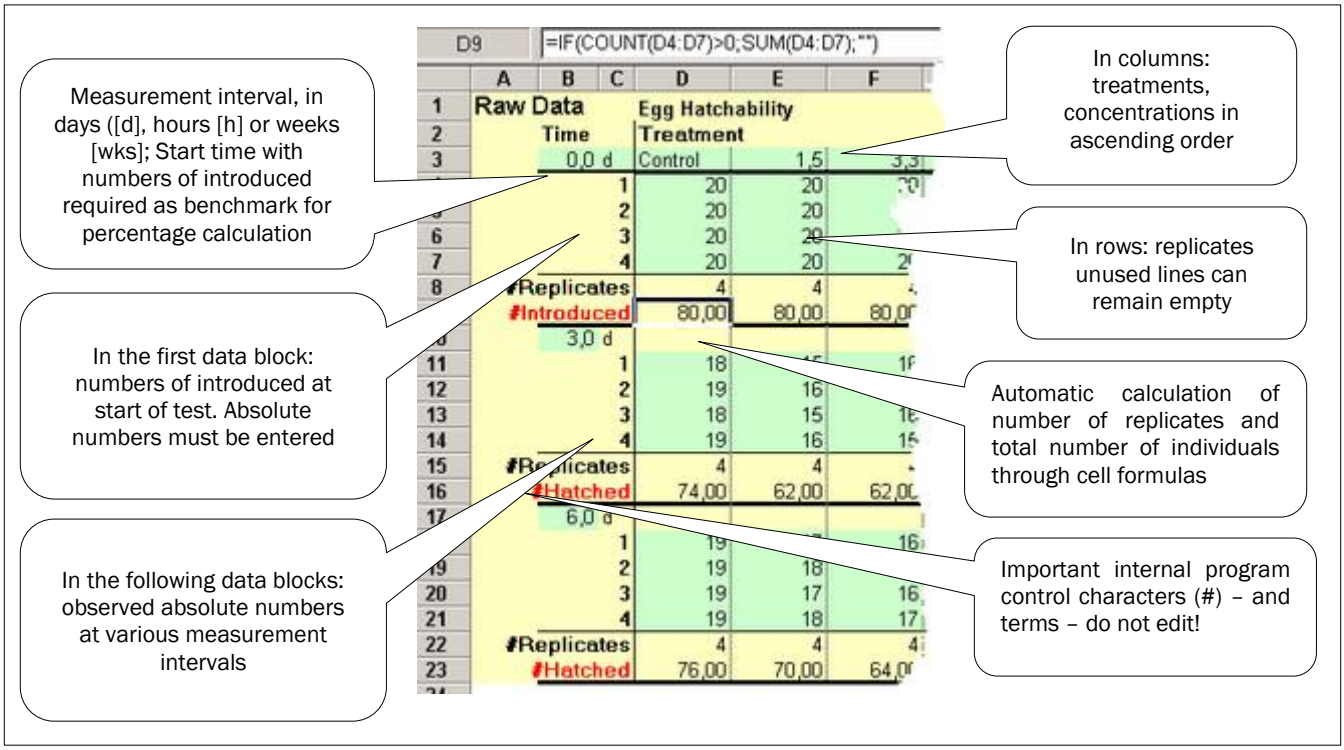


Figure 7: Data sheet (InputRawData) of a workbook – quantal data

Since with quantal data (Figure 7) basically percentages are calculated, a minimum of two measurement intervals must be shown: Number of introduced at the beginning of the experiment and the observed numbers at the measurement interval(s). The known measurement intervals as per the guidelines are pre-set in the biotest workbooks. These, however, can be edited. Also important: 2 out of 10 organisms are statistically not the same as 20 out of 100 organisms. Therefore, the absolute numbers must be entered (rather than percentages)!

Structure of a data sheet for metric data:

Example data files:
ToxRat Professional: see workbook OECD210, sheet Input Fresh Weight.
ToxRat Monitor und ToxRat Standard: see workbook *Testing a Metric response 3 at several Intervals.xls*, sheet InputRawData. Example: cell counts.

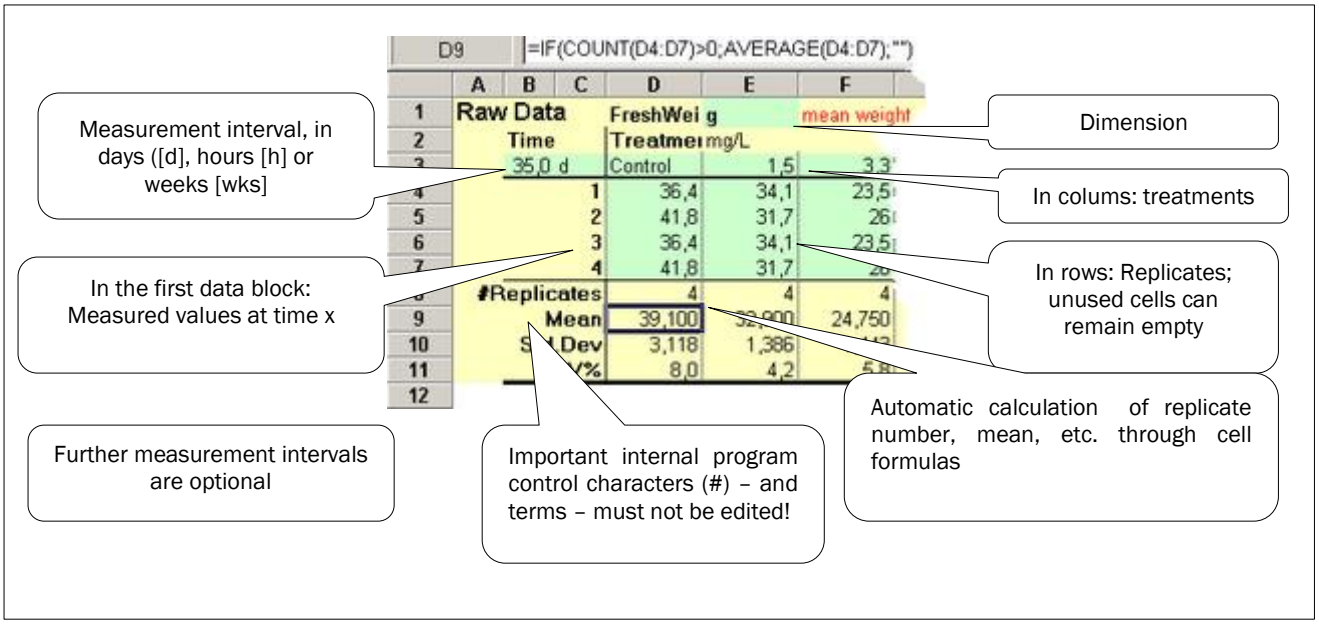


Figure 8: Data sheet (InputRawData) of a workbook – metric data

The number of measurement intervals for metric data (Figure 8) is flexible: The evaluation of the original variable is done for every existing measurement interval, regardless if it is just one or several. However, for the calculation of derived values such as yields or growth rates, a minimum of two measurement intervals is required. The existing measurement intervals in the biotest Workbooks have already been pre-set as per the guidelines. These can be edited. Whether derived quantities are calculated depends on the test guideline requirements, and is defined by default settings.
Only the original values for the entered data in the Generic Data Workbooks are evaluated. In the case where yields, growth rates, or other derived quantities should be statistically evaluated, these must be calculated by the user and the results must be entered directly as variable into the workbook.

Some calculations are already done in the data entry sheets by formulas in the appropriate cells: With quantal data, any replications will be summed up in the data sheets, with metric data, means, standard deviations and coefficients of variation will be calculated.
These calculations are done separately from later statistical evaulations, i.e. for the statistical evaluation in ToxRat all calculations are done internally new.
Hence the formulas in the data entry sheets have no effect on the statistical calculations. Their sole purpose is to give the user an initial overview of the possible tendencies in the dataset.

3.4 Filling in Workbooks or Masterbooks

You have now been familiarized with the fundamental structure of data entry templates. Now, nothing stands in the way of evaluating your data – the data must only be entered into a file. There are two possibilities for this:

- Data input directly using ToxRat
- Data input via MS-MS Excel

These options are valid for both Masterbooks and Workbooks. However, the following must be considered:

A Masterbook is only suited for data entry. In order to run an evaluation, the Masterbook must be changed over into a Workbook. This is done in **ToxRat** by pressing the „Refresh“ button (only visible when file is open).

In the case where your data is entered into a Workbook:

Do not forget: Whenever data in a Workbook is edited, this must be be read into the internal memory of **ToxRat**. This is done by pressing the „Refresh“ button (only visible when file is open).

In any case, the entered data must be saved – to do this, click on *File* in the Menu, and use either „save“ or „save as“.
Warning: „Save“ overwrites an existing file with the same file name without prompting! „Save as“ asks for a new file name and saves the file under a new file name and where requested, to a new location. For a list of the pre-set or recommended locations, please read Chapter 9.

Data entry within ToxRat

Enter the data directly into the corresponding green cells. Existing data can be simply overwritten. Some biotests have certain conventions on how to encode missing data (e.g. with a „-1“) – this is explained in the respective biotest workbook. Otherwise, as a rule, the applicable cells with missing data remain empty.
The C*opy* and *Paste* commands (Menu and Edit buttons or right mouse click) can be used, in order to enter large blocks of the same data, as an example. However, through this action, the cell format will also be copied over– this can be visually distracting. Therefore, we have the Workbookdesigner – which is an integrated tool in ToxRat, with similar formatting features offered as in MS Excel. The Workbookdesigner makes it possible to use ToxRat independently from MS MS Excel. Details to activating and using this feature are explained in the next section“.

Entering data via MS Excel

If the data already exists in an MS Excel file, it may save some typing time copying the information over into a ToxRat file. This is, in principle, possible using the Clipboard. However, by doing this, cell formatting will also be overwritten (e.g. colour coding). Moreover, what is even more important: copying data into ToxRat via clipboard causes cell protection, i.e. the cells cannot be edited afterwards without removing cell protection manually. This can be avoided by performing any copy-work exclusively in MS MS Excel (remember: ToxRat workbooks are xls-files!). To do this, ToxRat workbook or masterbook as well as the MS Excel source file must be opened in MS Excel, then data are copied by using the MS Excel commands „Paste - Only Values " from one into the other file. The filled in ToxRat workbook should then be saved as an xls file and opened in ToxRat to run the statistical evaluation.

ToxRat only processes MS Excel 2007 formats xlt and xls. When using a ToxRat template via MS Excel, please ensure that the file is saved with the extension xls, not xlsx.

3.5 Editing Workbooks und Masterbooks – Workbookdesigner

And what if the existing number of treatments, replicates, or measurement intervals in the template aren't suitable? Of course, the integrated templates can be adjusted to suit your individual test design needs. This is explained in this chapter.

Before you get started: The supplied templates are typically „oversized"– they can also be used for test designs with less replicates and test approaches. Cells that are not needed for replicates or measurement intervals as well as extra columns for treatments in a Workbook or Masterbook can simply be left empty. These do not need to be deleted – it is sufficient to clear any data that may already be pre-entered. Thereby data must be filled in without gaps, since data import into ToxRat is stopped at any empty column (treatment) or line (measurement interval). So surplus columns and lines must follow up the complete data set.
Nevertheless, the supplied templates may not cover all possible test designs. In these cases, treatments, replicates and measurement intervals can be added.

Whatever you do: Changes can be made exclusively in the data sheets (RawDataSheets) of a Workbooks or Masterbooks. No cells may be deleted or added in the cover page (General Notes)!

Analog to data entry, there are two possible options for editing workbooks and masterbooks:

- Editing of templates in ToxRat
- Editing of templates via MS Excel

Regardless of which tool you are using, the following applies: The yellow cells in the data sheets contain formulas, which must not be overwritten. Therefore the data sheets are protected.

To change a masterbook or workbook to suit your test design, the worksheet protection must be de-activated.

The ToxRat software includes the Workbookdesigner – a tool, which offers similar formatting options as in MS Excel. The Workbookdesigner can be found under menu item *Edit or* directly by clicking on the corresponding button (Figure 9):

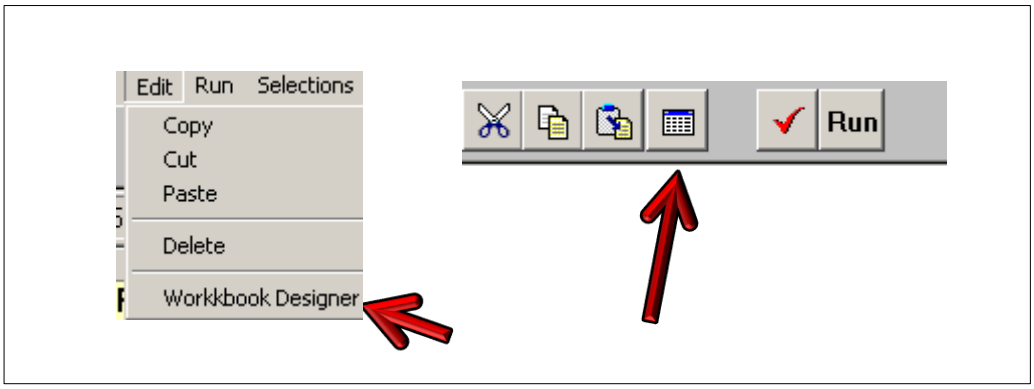


Figure 9: How to activate the Workbookdesigner

The workbookdesigner opens the current file in a separate window (Figure 10) and displays several commands to enable copying, deleting, and pasting of cells and cell contents, just as you know it in MS Excel.

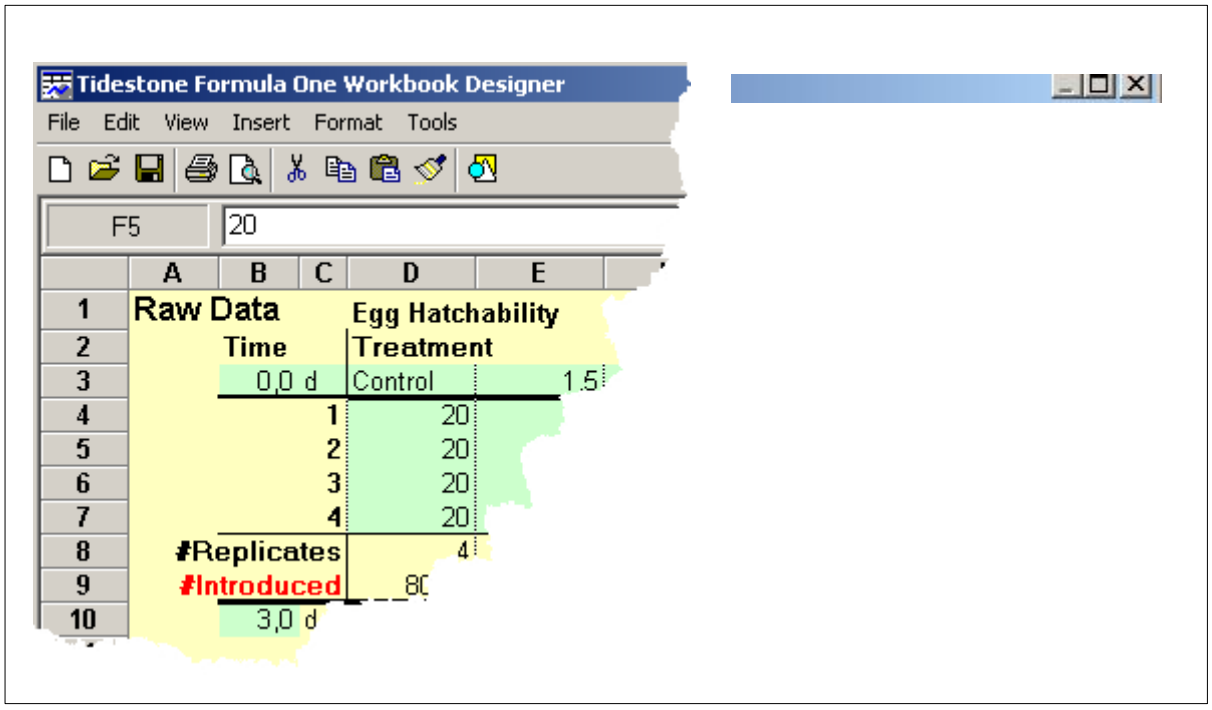


Figure 10: user interface of the Workbookdesigner

In order to edit a data sheet, the worksheet protection must be de-activated – this can be done by clicking on the commands Format-Sheet-Protection (Figure 11).

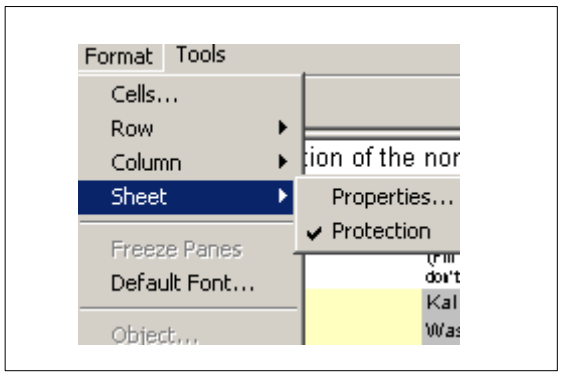


Figure 11: De-activating worksheet protection in the Workbookdesigner

The further menu items and commands in the Workbookdesigner correspond to the ones known from MS Excel and therefore will not be discussed here. You will mainly need the commands to copy and paste rows and columns.

When pasting additional test approaches (treatments), it is best to copy the contents from an existing column into an empty column to the right of the existing data block. With this you will automatically assume the corresponding cell formulas for the calculation of sums, averages, etc.

When pasting replicates (rows), you must ensure that this is done in the same manner for all measurement intervals, so that in the end, the number of replicates for all measurement intervals is the same. Important: Please make sure that when pasting rows into the individual measurement intervals, the cell references for the formulas for each of the sums, averages, and standard deviations, etc., get updated. Complete measurement intervals can be added on to by copying the existing block and pasting it below the last existing measurement interval.

By clicking on the cross at the top right of the menu bar, you will exit the Workbookdesigner and return to the user interface of ToxRat.

The Workbookdesigner allows to use ToxRat completely independently from MS Excel. However, if you are more familiar with MS Excel it may be more comfortable for you to make the necessary workbook adjustment in MS Excel. The above-mentioned directions for deactivating the data sheet protection and updating formula references can also be applied here.

4 The Evaluation

4.1 Menus and Buttons on the Evaluation Screen – Start Evaluation

As soon as you have opened a Workbook, new menu items and buttons on the menu bar for evaluating are available. These are also content specific and the ones that are available depend on if you have opened a Bio Test Workbook or a Generic Data Workbook.

4.1.1 The Evaluation Screen in Biotest Workbooks

Our example files are:
ToxRat Professional: Workbook OECD210
ToxRat Monitor: Workbook DIN EN ISO 15088

Figure 12 shows the new menu in a Biotest Workbook.

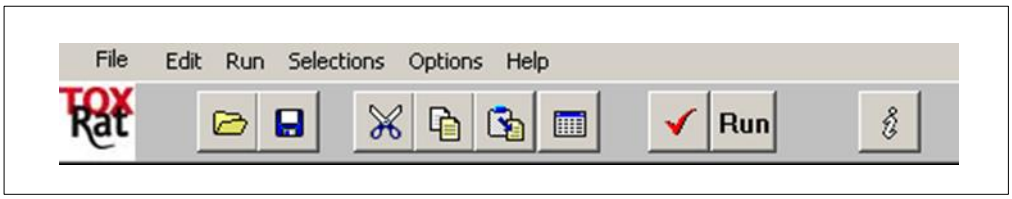


Figure 12: Menu on the Evaluation Screen– Biotest Workbook

Newly added commands for the evaluation are

The "brain" of ToxRat !
Starts the evaluation. More information is available below.

„Refresh" – re-loads the entire file from new;
This is necessary after new data has been entered, or after settings have been changed in the various menus (in these cases, you will see „changes require clicking the refresh button"). Existing evaluations will be deleted through the "refresh" action. Important: Previously made Settings under Options will remain, except settings under „Selections" – these will be reset to „default settings" (see Chapter 4.2).

Here you determine which variables, measurement intervals, and test approaches will be used for the next evaluation sequence when RUN is selected and if the data should be transformed. More details in Chapter 4.2.

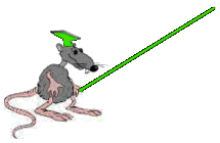
Under Options, new menu sub-items are available to select certain statistical methods other than the program default settings. You can find out more about this in Chapter 4.3 and in 4.7.

The most important new command on the Evaluation Screen is the command:

With the RUN command you start a complete evaluation sequence. Program settings will be used for this process. You can follow these expert recommendations, but you don't have to. It is recommended to first get an overview of the data with the standard evaluation – and then change certain individual methods as desired.

Depending on the type of biotest performed (i.e. the associated guideline), clicking RUN will trigger relevant evaluation types that are batch processed. Important to know: RUN will always generate the results required by the associated guideline, including validity checks. In our OECD 210 example (Fish Early Life Stage Toxicity Test), clicking RUN will determine ECx values and NOECs for multiple variables and measuring times between 3 and 35 days, followed by a validity check as required by the test guideline.
Try it now! An explanation of the results ("What do I find where?") can be found in chapter 6. Let's delete these results for now by clicking "Refresh". Chapter 4.3 elaborates on the statistical methods automatically applied for biotest analyses, and explains how to edit the preconfigured selection.

For the example DIN EN ISO 15088 (Acute Toxicity of Waste Water to Fish Eggs of Danio rerio) data for only one variable (mortality) for two measurement intervals (24 hours, 48 hours) is available. The EC values and LID (lowest ineffective dilution) will be calculated based on the corresponding guidelines and a validity check will be run. Here also applies: Just give it a try!



„Refresh" reads changed settings or data. The temporary program internal memory will be cleaned. This can be very helpful, when the memory is full after running several evaluations of the same file to avoid undesired output or that the program "freezes".

4.1.2 The Evaluation Screen in Generic Workbooks

Our example file for all ToxRat programs is Workbook „Testing a Metric response 3 at several Intervals.xls“.

Figure 13 shows the new menu when a Generic Data Workbook is opened.

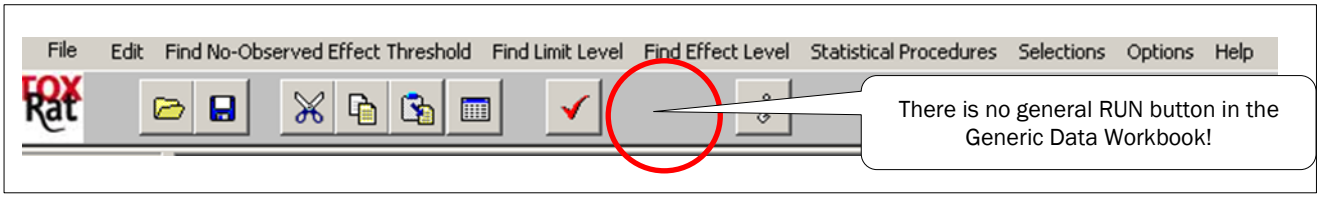


Figure 13: Menu Evaluation Screen – Generic Data Workbook

The following buttons and commands are the same as in the Biotest Workbooks:

„Refresh“ – see explanation for Biotest Workbooks; the same applies here.

See explanation for Biotest Workbooks; the same applies here.

Additionally, new menu items are now available, with which specific statistical methods can be selected directly (“Find no Observed Effect Treshold”, “Find Limit Level”, “Find Effect Level”, “Statistical Procedures”). More to this in Chapter 4.3.2.

Before you choose a statistical method, you should determine which data should be evaluated. For this, we will now look at the menu item „Selections“ in the next chapter.

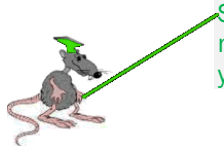
4.2 Which data should be evaluated? The „Selections“ Menu

Or: Selection of variables, measurement intervals, treatments, transformations

Regardless if your data exists in a Biotest Workbook or in a Generic Data Workbook and which statistical process you wish to use, you can determine which data in the Workbook should be evaluated at all. This means, which variables and which measurement intervals you need, or if certain treatments should be temporarily excluded from the evaluation or if the data should be transformed. For this, you will use the menu item „Selections“. Important: All existing settings are optional – if you don’t select any settings, then ToxRat will use the default settings.

The necessary settings for the evaluation in Workbooks have already been pre-set in the Selections menu („initial state“). Therefore, you will not need the “Selections” menu for standard evaluations!

Also important to know:
The settings that you choose in Selections will only be maintained for the next following evaluation. As soon as “Refresh” or „Restore Default Settings“ is clicked, a new evaluation with the RUN button is started, or if the Workbook is re-opened again, all settings under Selections are restored to the standard values.
This is done on purpose to ensure that certain data is not accidentally excluded from an evaluation.



Settings under“Selections” are only temporary. This means, that they only apply to the next following evaluation. Therefore, it is recommended to select your settings just before you run the evaluation.

Figure 14 provides an overview of the set-up options in the Selections menu.

Please use the following example files:
ToxRat Professional: Workbook OECD210
ToxRat Monitor: Workbook DIN EN ISO 15088
ToxRat Standard: Workbook „Testing a Metric response 3 at several Intervals.xls“.

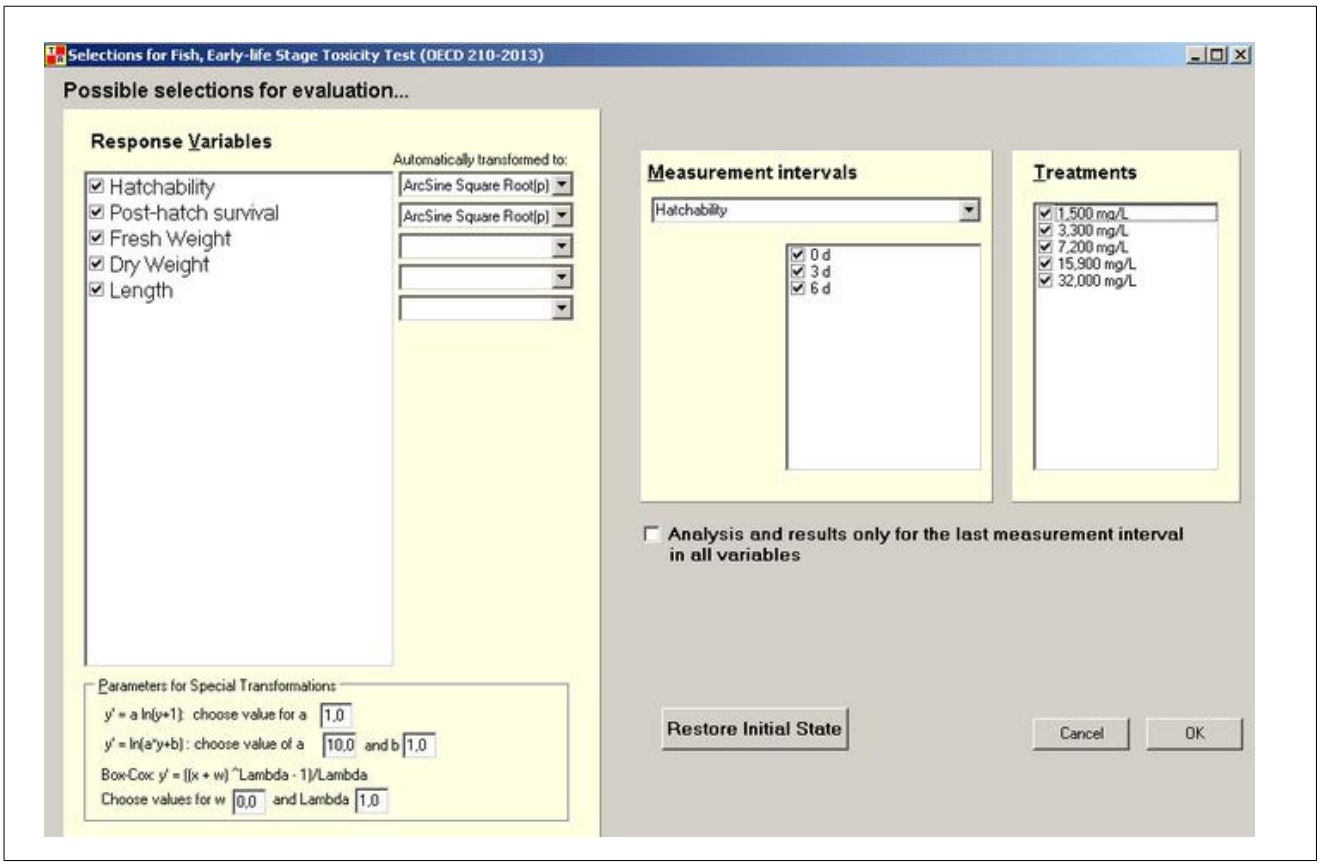


Figure 14: Menu „Selections"– Options for Biotest Workbook OECD 210

Response Variables

All the variables, which can be evaluated with the current Workbook, are listed here. The variables that will be used in the next evaluation have been pre-selected. There is basically only one in the Generic Data Workbooks, however, in the Biotest Workbooks, the list can be quite long. If you don't need all of them, then you can temporarily deselect specific ones.

Data Transformation

Sometimes it makes sense to transform the original data, whether it is to evaluate quantal data with parametric tests or whether it is to bring normality and variance homogeneity to metric data.

For metric variables, you can select individual functions for each metric variable and individually match them where required (see Figure 15). These will then be used in the next evaluation.

For quantal variables, an arc-sinus transformed parallel data record is kept in the background. It will only be used if you choose a parametric test for determining NOEC – this does not happen in this menu, but in the Options menu (see Chapter 4.4.2).

Since the program does not know at this point which test you will select (a parametric or a non-parametric one), the arc-sinus transformation is always shown in the Selections menu for quantal variables. You don't have to – and you can't – create any settings here!

With default settings, ToxRat always uses the untransformed original data for both metric and quantal variables. If you select a data transformation, the used function will be shown in the results table of the corresponding variable.

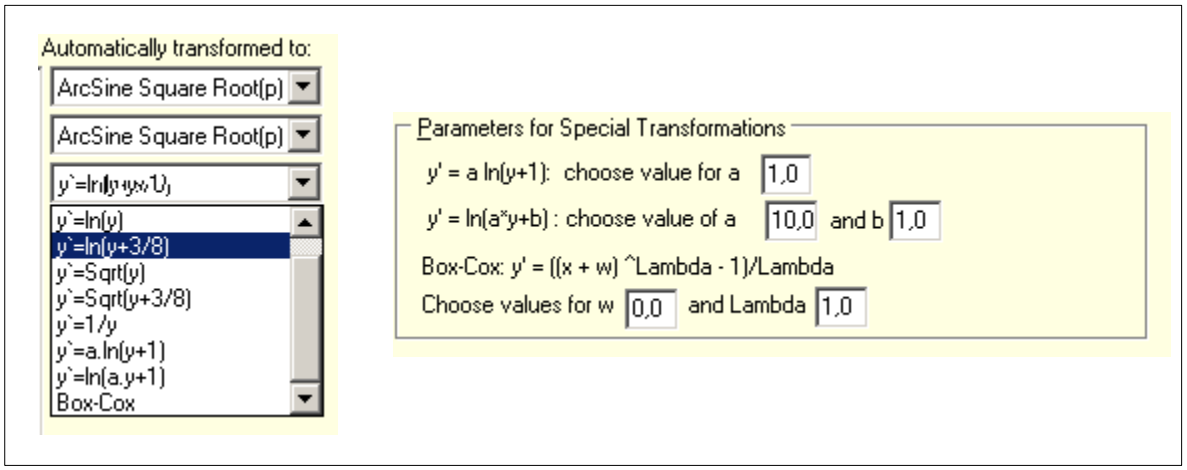


Figure 15: In the Selections menu selectable data transformation for metric data

Measurement Intervals

If several measurement intervals exist, then you may exclude or include individual ones from the next evaluation. This can be set for each variable individually by selecting the corresponding variable in the pull down menu at the very top of the screen.
In order to make your job easier for you, the commonly used setting „Only last measurement interval for all variables" can be selected with a simple check mark.

Treatments

The selection window „treatments" enables you to test, how excluding individual treatments affects the results of the calculations. This can be very useful if, for example, the result of a specific treatment is supposed to be erroneous due to experimental reasons. You can then run a trial evaluation excluding this treatment, without having to delete the corresponding raw data. Of course, by default, all existing treatments for evaluating are included in evaluation.

4.3 Selection of the Statistical Method

Regardless if you use ToxRat Professional, ToxRat Monitor or ToxRat Standard: All ToxRat programs include the same statistical methods! However, the operating options differ depending on the type of workbook used.
ToxRat provides expert knowledge, which statistical method should be used with a certain data set, resulting in certain default settings for evaluation. You can follow these recommendations – or you can replace them with your individual settings. In this chapter, you will learn where the corresponding menus can be found, how you can control and save your settings and if necessary, how you can re-set the default settings.

4.3.1 Selection of Statistical Methods in Biotest Workbooks

You have already seen that you can run an evaluation without once having to select or confirm a single statistical method yourself – simply by clicking on the RUN button and letting ToxRat choose the methods. For this, ToxRat uses default settings, which in every case guarantee a correct evaluation of your data. Nevertheless, you or your sponsor may prefer a different method and/or the data requires to try an alternative method. In this case, you have the option to change the program settings („default settings") with your individual settings („user settings").

Please use the following example files:
ToxRat Professional: Workbook OECD210 , ToxRat Monitor: Workbook DIN EN ISO 15088

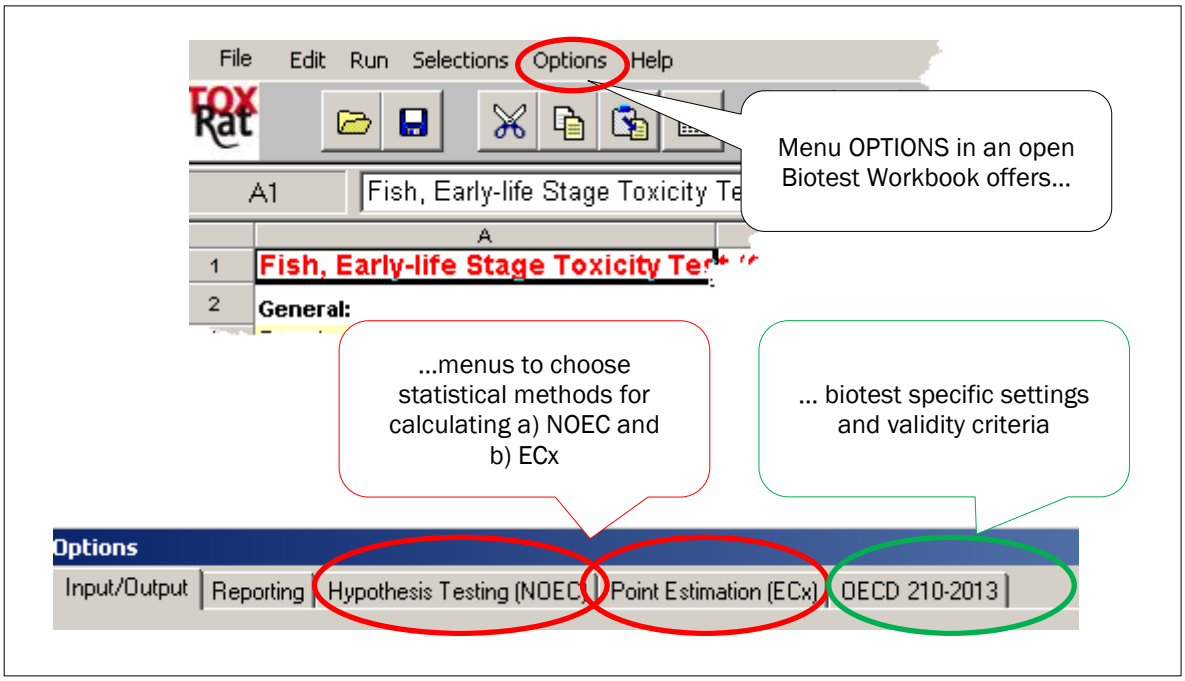


Figure 16: New "Options" menu content if a Biotest Workbook is opened.

In order to select statistical methods, you need to select the „Options" menu. You should already know this menu item from the start-up screen, however, it is context sensitive and therefore now - in an opened Biotest Workbook - additional options are available (Figure 16).
In the menu options, you will find a tab with biotest name, offering test-specific settings and validity criteria (here: OECD 210). You will also find methods for calculating effect thresholds („Hypothesis Testing NOEC") and dose response relations ("Point Estimation ECx"). Which statistical methods are at this point available, how you choose them, and which settings are to be selected, will be explained in Chapter 4.4 to 4.7.

4.3.2 Selection of Statistical Methods in Generic Data Workbooks

Use the following example files: „Testing a Metric response 3 at several Intervals.xls".

The „bad" news is: there is no RUN command!
This is due to the fact that a Generic Data Workbooks is not tied to a specific guideline and therefore no program settings are set up to choose which statistical endpoint should be evaluated. The „good" news is: Instead, a new menu item is available such as „Find-No-Observed-Effect-Threshold", "Find Limit Level" and „Find Effect Level" (Figure 17).

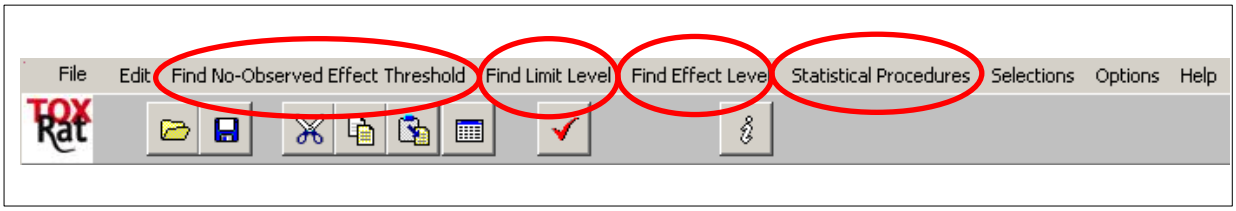


Figure 17: Menu commands for statistical evaluation in Generic Data Workbooks

With this, you can „instruct" ToxRat to run a corresponding evaluation using program settings. This means, you don't have to decide yourself, which statistical methods or settings should be used, instead, you can rely on the integrated statistics expertise. When selecting „Find No Observed Effect Threshold"- and „Find Limit", you will receive a NOEC and Limit Concentration from a sequence of pretests and an adequate final test. Of course, you can change the default settings with your own choices at any time (see section 4.4 and 4.5).

As of version ToxRat 3.0, you can run a complete evaluation sequence of pretests and final test for NOEC calculation also in the Generic Workbooks – a RUN button for this is available in a corresponding dialogue box.

By selecting „Find Effect Level", ECx values will be calculated. Thereby, also default settings alre availbale. However, it is on you to check, whether the results are reasonable. Possibly you have to select different settings – for assistance please see section 4.6).

Before we will take a look at the associated selection windows in more detail, as a next point, we'd still like to introduce you to the menu item „Statistical Procedures", which is also available on the evaluation screen for Generic Workbooks (Figure 17).
In contrast to the „Find xyz" menus, which trigger complete *test sequences*, „Statistical Procedures" offers specific statistical methods *separately*, therefore you can choose and run individual statistical methods (
Figure 18). Simply select the different menu items in order to see which statistical method is available.

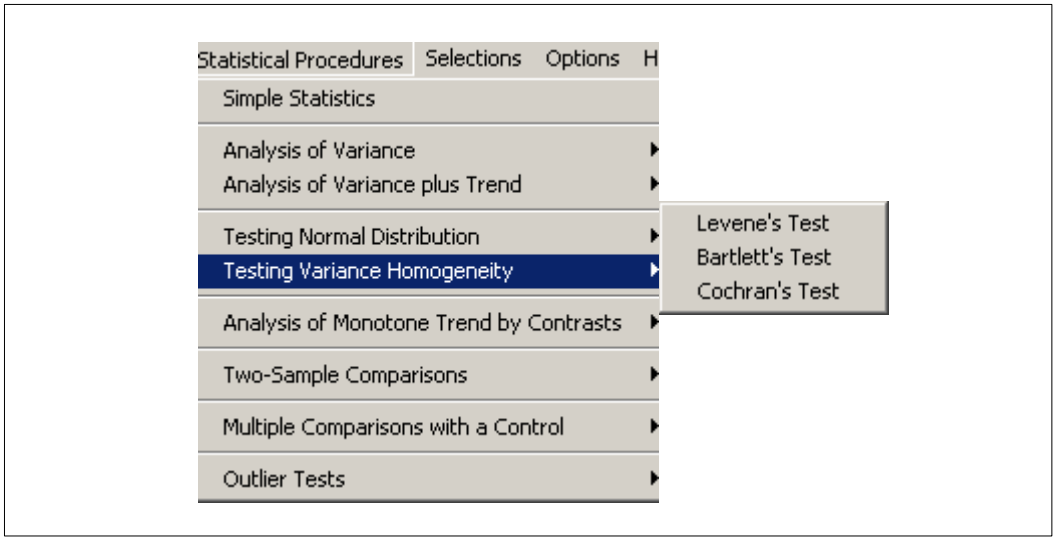


Figure 18: The menu „Statistical Procedures"

The content of the pull-down menus „Statistical Procedures" are also context specific, and therefore, depending on if you have a Workbook for quantal or metric data open, ToxRat shows you only the methods for the specific variable type. The individual processes will be introduced and discussed in Chapter 4.7. – here we´d like to introduce you to the general operation.
As a rule, after selecting a procedure, a dialogue window with further settings opens (
Figure 19). By clicking on the "Radio" buttons, you select the "Significance Level", the „Test Direction" and – with some parametric tests – „Which Variance to Use". You don't have to make any settings here – if you don't change anything, the default settings will be used. The pretests for normality and variance homogeneity will be run with the recommended value for the signifance level of 1% (0.01). All other tests are set at the standard signifance level of 5% (0.05).
Some tests are always run two sided. In these cases, the corresponding check box is de-activated, and can therefore not be changed. Otherwise: for metric variables the default test direction is „one sided smaller" and for quantal variables such as mortality, „one sided greater". These can be adjusted based on your individual needs.
For two samples or multiple t-Tests, you can decide if the residual variance from an ANOVA or the individual control and treatment variances should be used. The default setting always is the more powerful variant. For most of the parametric tests, the type of variance to be used has been pre-set and therefore the check box is de-activated.

All of the defined conditions of a test in the dialogue window will be shown in the corresponding legend of the results table. A click on „OK“ starts the evaluation.

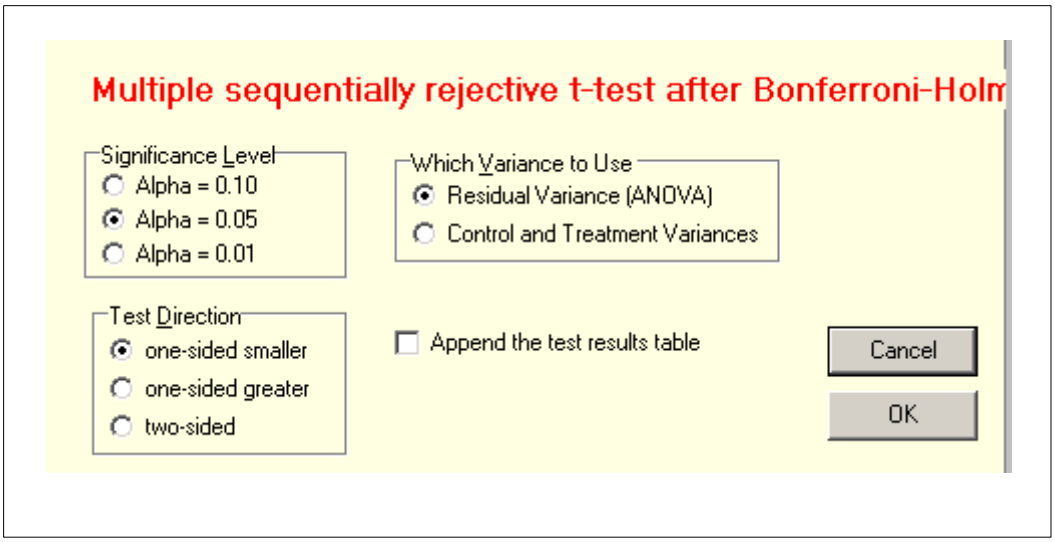


Figure 19: Dialogue window after selecting a statistical process

In order to make your job easier, we have made the control box „Append the test results table“ available. If it has not been selected, then the results from previous evaluations will be deleted and replaced with the current data for the next evaluation and you will only receive the results of the currently selected evaluation. If „Append the test results table“ has been selected, then the results for the next evaluation will be added to the table with the already existing results.

If the „Append the test results table“ has been checked, then the next results for the current data set will be added to the pevious ones. With this, you can accumulate and save the results from various statistical tests in one file, and present them in an overall report.
Otherwise, previous results will be deleted and only the results of the currently selected process will be shown.

4.3.3 Checking and Saving Settings, and Restoring Default Settings

How to keep track of the many settings?

The settings, with which a specific bio test is evaluated (pretests and final tests, significance levels, test direction, validity criteria, etc.) will be summarized in a so-called „Settings“ table and – optional – listed together with the results. This enables you to go back at any time and check if and which default settings were changed. You will learn more about this in the discussion on results display in Chapter 6.5.2.

How does ToxRat know which settings should be used for certain evaluations?

The settings, which ToxRat should use, are saved in so-called „Settings“ files. A „Settings“ file exists for each Workbook type (e.g. set_OECD210.stp). Whenever you open a Workbook of a certain type, ToxRat reads the settings in the corresponding “Settings” file. Initially, these are the default settings.
Perhaps you routinely run a certain bio test with specific settings for the statistics that deviates from the default settings in ToxRat. Then it would be quite bothersome if you had to change the settings again every time you needed to run the test anew – in particular if it deals with a Workbook with many different variables.
For this purpose, your individual user settings are saved. Whenever you save a Workbook including results sheets, the current menu settings, with which the results were generated, are saved to the corresponding “Settings” file. Thereby, default settings will be overwritten. The saved settings, are automatically available again as soon as a Workbook of the same type is opened, e.g. again a biotest as per OECD 210, even if it deals with different data (e.g. if it is a different file).

Overwritten default settings –what to do?

Whatever you choose to change in the settings: You can restore the recommended default settings at any time by simply clicking on „restore default settings“.
The button „restore default settings“ can be found at the very bottom of the Options menu (Figure 20). It is located here, because it applies to the entire workbook – thereby there are two levels of “restoring”:
If a workbook has been opened, “restore default settings” restores the ECx- and NOEC-settings of all variables and all statistical methods of the current workbook. In contrast, all settings in “Input-output” and “Reporting” (see Figure 90 and Figure 94) remain permanent for all workbooks unless again changed by the user.
If no workbook has been opened, “restore default settings” restores all settings in “Input-output” and “Reporting”.

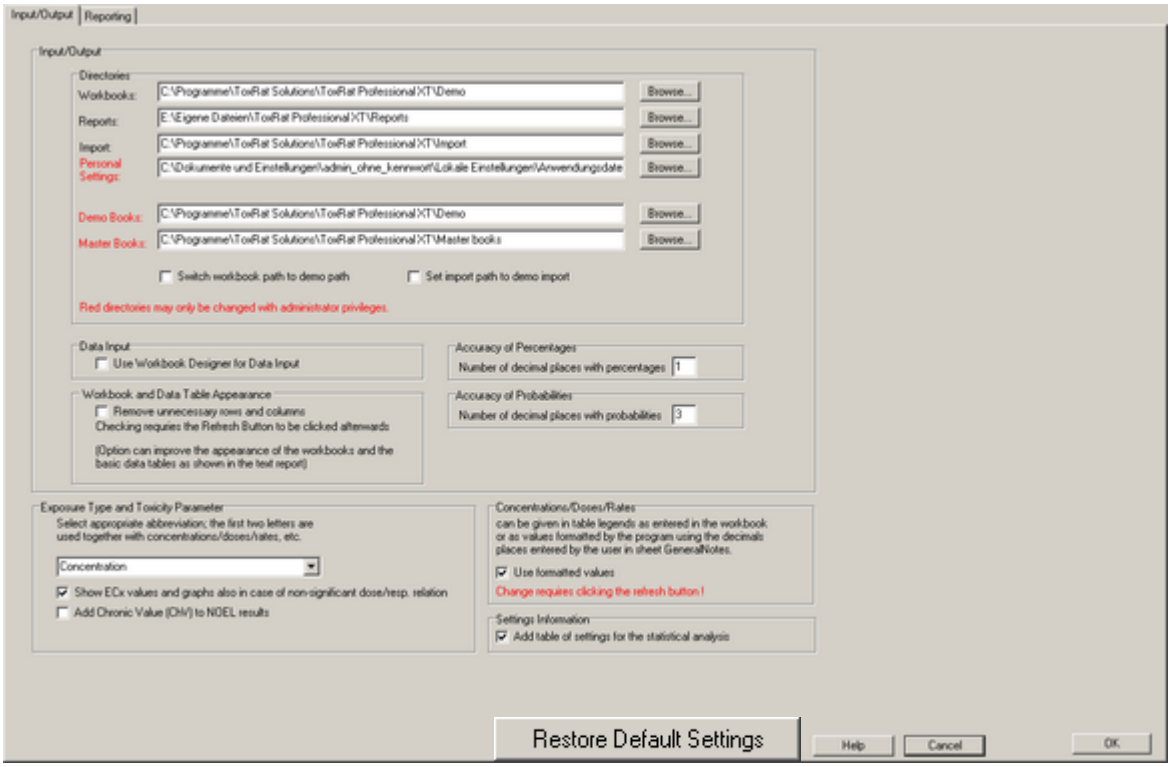


Figure 20: Button „Restore Default Settings“ in the Options menu

„Restore Default Settings“ restores all settings for the current Workbook type with a single click to the initial settings.

ToxRat lists both the results and the settings, being used to obtain these results in a table. This enables you to look back at which settings were used to run the evaluation. Additionally, the settings will be saved in a Workbook specific “Settings” file and automatically re-used, as soon as you open a Workbook of the same type (unless you click “restore default settings”)..

We recommend that at first, you evaluate and analyze a file with the default settings, and then, as necessary, change to individual settings. Conclusion: Better once too often rather than once not enough „restore default settings“!

The „Settings“ files are saved in a certain user-specific directory (see Chapter 9,, Installation). This allows individual users to use ToxRat with their own settings even if several users work with the program. If a Workbook is opened, for which no corresponding „Settings“ file is available (yet), then ToxRat applies the default settings.

4.4 Process for NOEC Determination (Multiple Testing)

Certain criteria exist when selecting a statistical test for deriving a No-Observed-Effect-Concentration (NOEC):

- Variable type (metric or quantal)?
- Is the data replicated?
- Does the data show normal distribution?
- Does the data show variance homogeneity?
- Does a monotone dose response relation exist?

Figure 21 and Figure 22 each show a general diagram for the test selection. You can use this diagram to run a NOEC determination manually.
In the automatic evaluation mode, ToxRat follows this test diagram step-by-step, i.e. an appropriate method is selected depending on the output of the previous tests performed. Finally, the most suitable multiple test for determining the NOEC is selected.

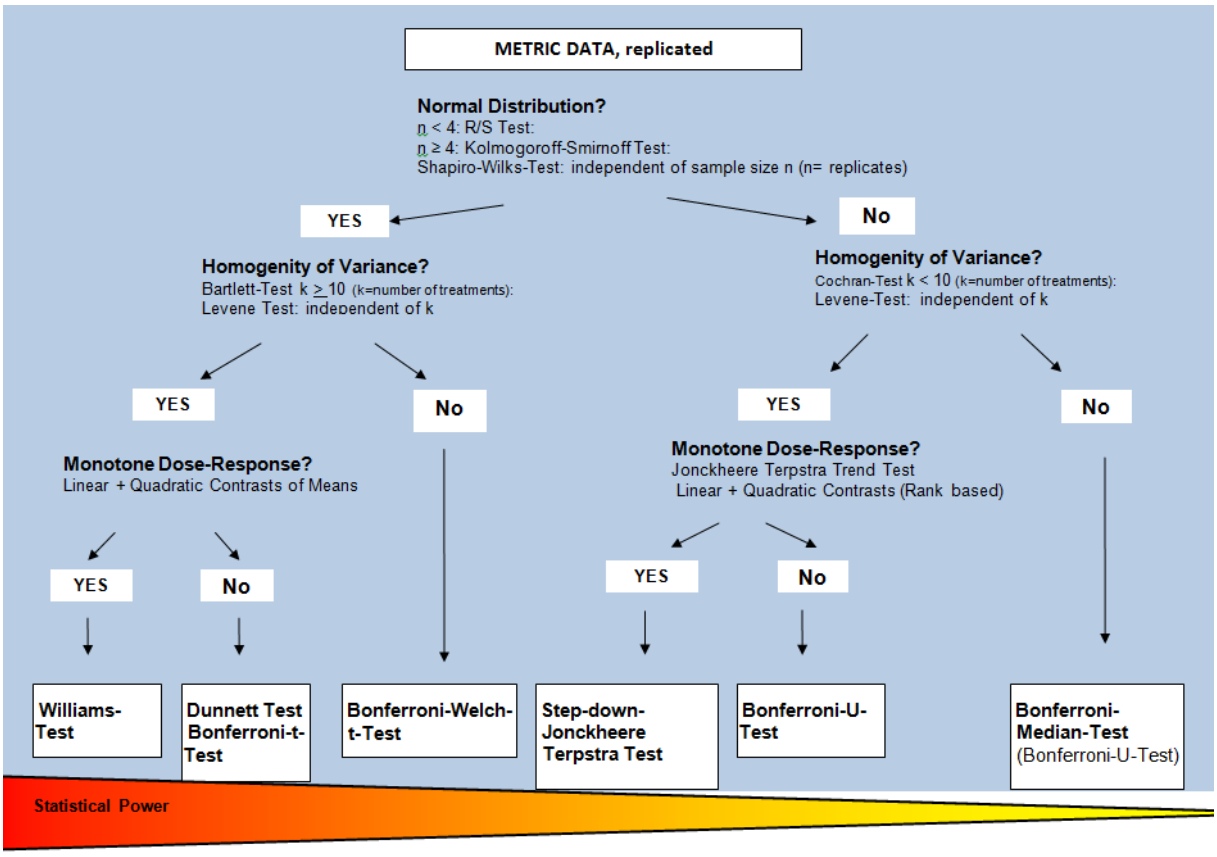


Figure 21: Test schema applied in ToxRat for determining a NOEC for metric data

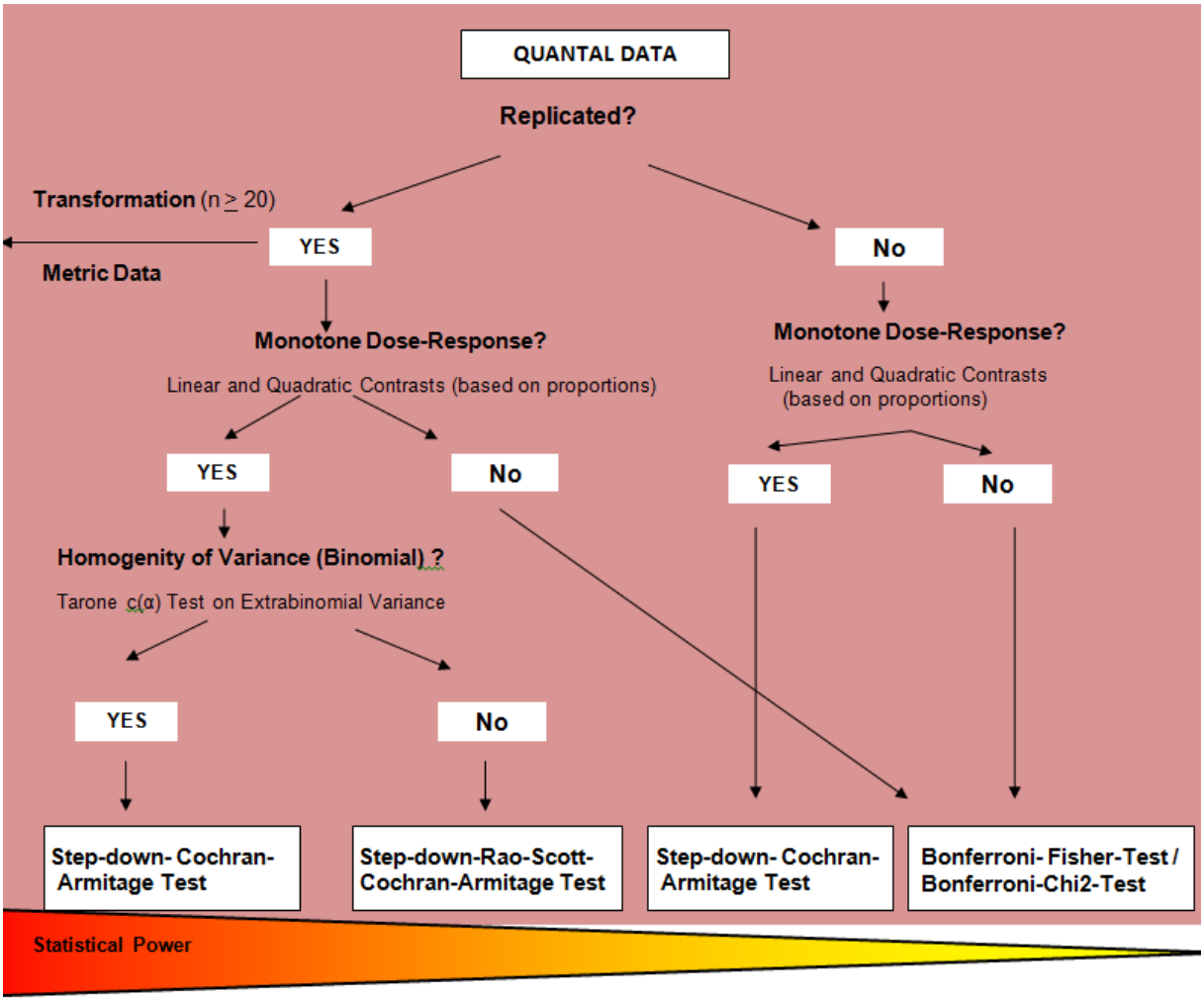


Figure 22: Test schema applied in ToxRat for determining a NOEC for quantal data

As of version ToxRat 3.0, the expert evaluation mode for NOEC determination (i.e. automatical selection of appropriate final test depending on pretesting results) is available also for Generic Workbooks– A RUN button is available in the corresponding dialogue box.

The available multiple tests for determining NOEC arranged by statistical power can be seen in Figure 21 and Figure 22: The power decreases from left to right.
As a general rule: when a specific criterion has been not fulfilled (e.g. normal distribution), you must not select further processes from this track. In contrast, if normal distribution is fulfilled, you are allowed to select further processes from this track – but you don´t have to. From a statistical point of view, it is not forbidden, for example for a normally distributed set of data to select a test that doesn't require normality. However, with this, one loses out on statistical power.

In the automated evaluation mode ToxRat always selects the most powerful test possible for the given data type and with the untransformed original data. You can, of course, change the default settings and fix a certain pretest or final test, or add further optional tests. The corresponding dialogue windows will be explained in the following sections.

With the generic workbooks, all tests shown in Figure 21 and Figure 22 are available for individual manual evaluation – for more details please see Chapter 4.7. First we will show you how to manage ToxRat´s automatic evaluation mode.

4.4.1 NOEC Determination for Metric Data

Please use the following example files:
ToxRat Professional: Workbook OECD210
ToxRat Monitor: Workbook DIN EN ISO 10706
ToxRat Standard: Workbook „Testing a Metric response 3 at several Intervals.xls“.

If you have opened a Biotest Workbook, then please use the menu item „Options -– Hypothesis-Testing (NOEC)“. If you have opened a Generic Workbook, please use the menu item „Find NOEC“(compare Chapter 4.3). A window will open (Figure 23), with all the procedures available which are shown in the test diagram for NOEC determination for metric variables (Figure 21). The window is identical for both Biotest Workbooks and Generic Data Workbooks, with three exceptions:

1. The green variable check box (“Select variable for which adjustment applies“), is only available for Biotest Workbooks, since only there several variables that can be evaluated at the same time.
2. The box „Limit Testing / Two Sample Testing“ is only available in this menu in Biotest Workbooks; in Generic Data Workbooks it can be found under its own menu item „Find Limit Level“ (with identical content). We will discuss these methods in Chapter 4.5 , „Procedure for Determining a Limit Concentration “.
3. In Biotest Workbooks, you will confirm your selections in this dialogue window with „OK“(below right). Subsequently, you will start the complete evaluation sequence with the RUN button in the evaluation window (compare Figure 12). In Generic Data Workbooks, you will confirm *and start* the selected settings with the RUN button (below right).

If several variables exist in a Workbook, then each variable will have its own standard settings. If you would like to change these settings, you must (and can) do this separately for each variable.

For users of ToxRat Professional and ToxRat Monitor: First enter a metric variable in the green variable selection box. OECD 210: “Fresh Weight”; ISO 10706: „Cumulative Offspring per Survivor“.

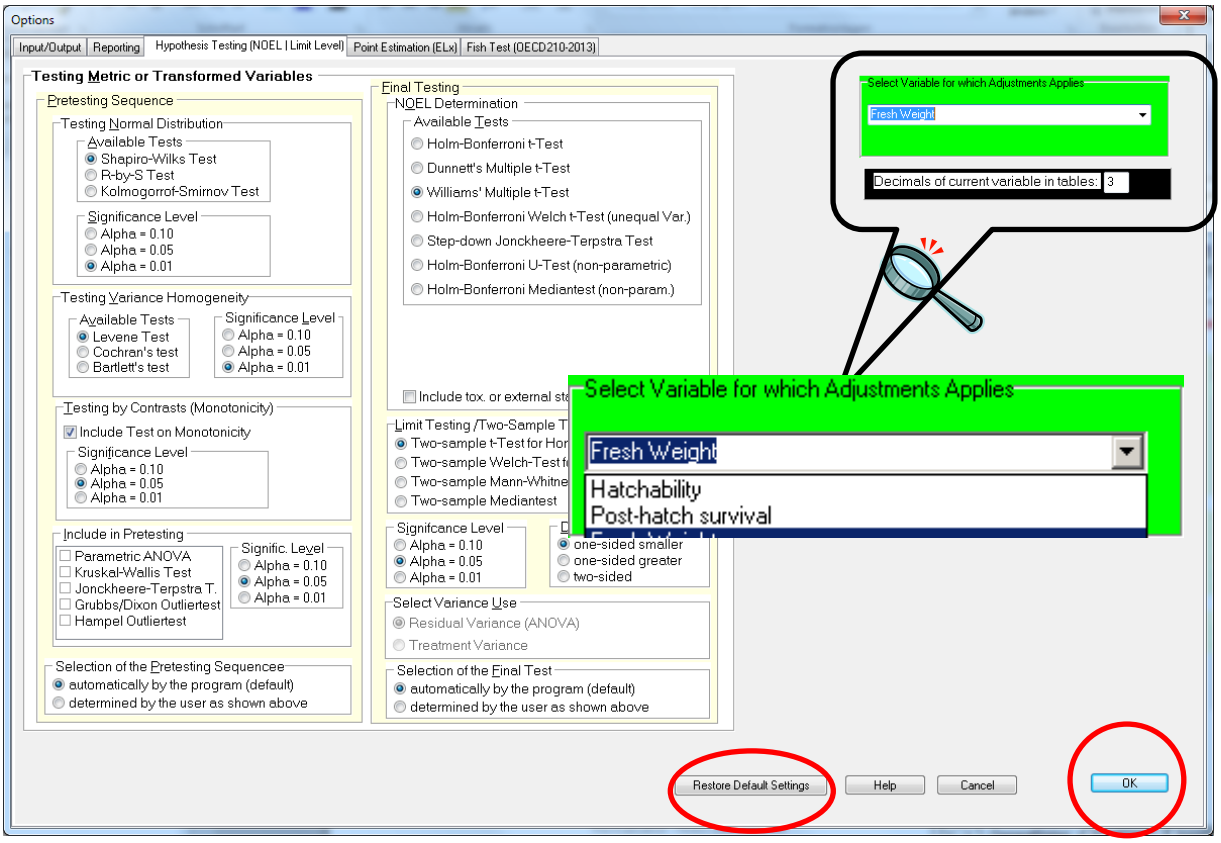


Figure 23: Dialogue window NOEC determination for metric data

Let´s have a closer look at the dialogue box „Testing Metric or Transformed Variables“ (Figure 24). The left side deals with pretests („Pretesting Sequence“), the right side deals with final tests („Final Testing“).
Both sides conclude with a box, in which you select if ToxRat decides on the test sequence („automatically by the program“) or if your own settings should be used („determined by the user as shown above“). Only if the latter is selected, your own personal settings will be applied!

Your own settings will only be applied once you have clicked on the „determined by user as shown above“ button. Otherwise, ToxRat always uses its internal standard settings, regardless of what is selected in the NOEC selection window.
Attention: this only applies to the mandatory testing, for which ToxRat recommends the default settings. Additional processes and settings („Include in pretesting“, „Include toxic or external standard“…) can also be added under the automatic mode.

Default settings are available for the mandatory procedures for NOEC determination – these can be restored at any time with the click of a button „restore default settings“ (Figure 23, bottom left).

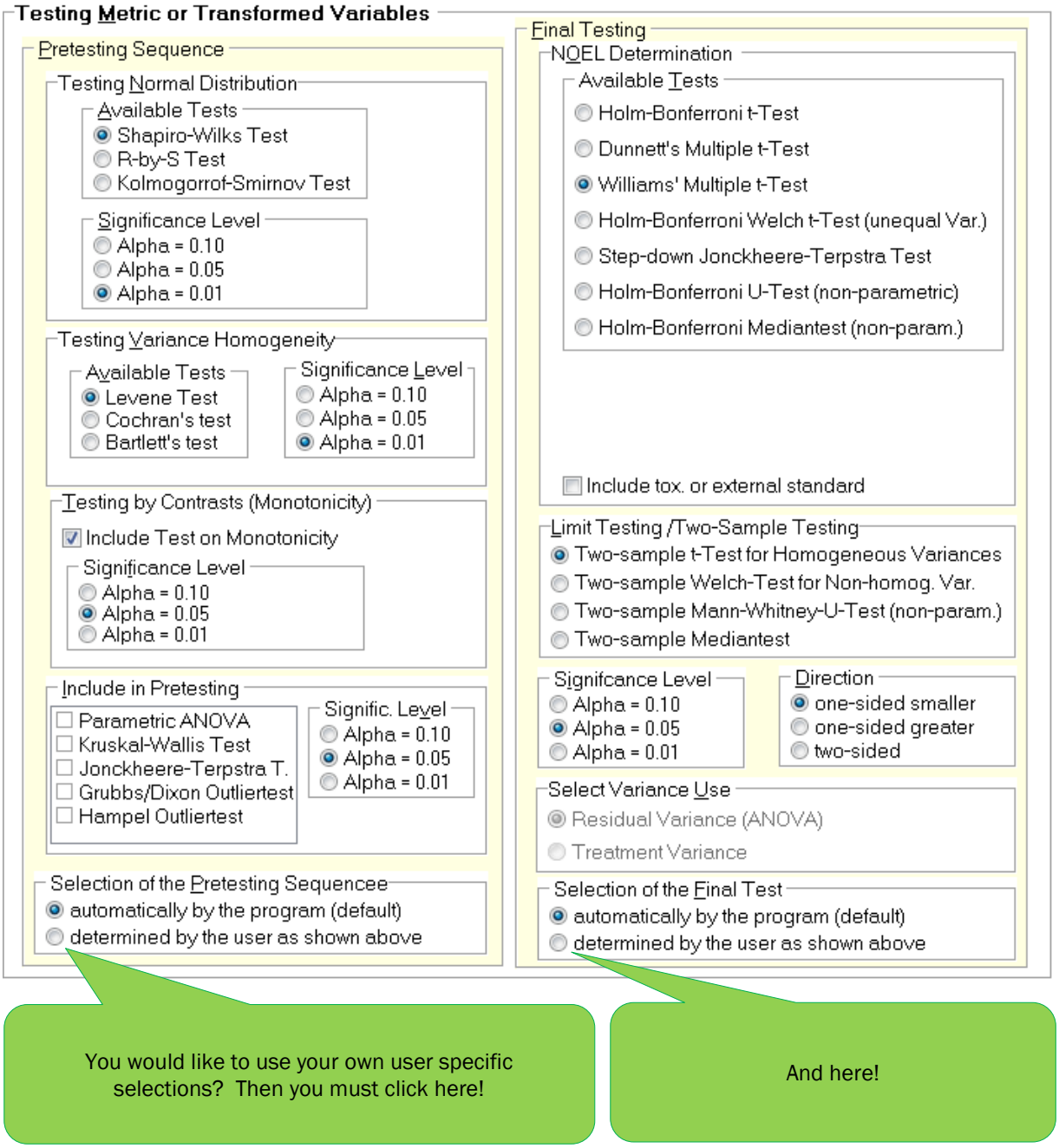


Figure 24: Dialogue window „Testing Metric or Transformed Variables“ for NOEC determination, standard settings

There are various groups of **pretests** corresponding to the selection criteria for a suitable for NOEC determination (please see Figure 21):
Normality tests (Shapiro-Wilks Test, R/S-Test, Kolmogorrof-Smirnov Test), Tests on Variance Homogeneity (Levene Test, Cochran´s Test, Bartlett´s Test) and Monotonicity Test (linear und quadratic contrasts), each with their individually selectable significance level. These tests are always performed two-sided and therefore the test direction cannot be set.

ToxRat prefers the Shapiro Wilks Test and the Levene Test as the tests for normality and homogeneity variance because they check the entire data as a whole to produce a clear result and because they do not depend on sample size and number of treatments.

Since normality and homogeneity of variance are fundamental prerequisites for the use of powerful final tests, a significance level (error probability) of 1% is recommended for the pretests, to reduce the risk of a false-positive result (and therewith unnecessary weak final tests) and to prevent alpha-cumulation (= cumulation of single error probabilities in a test sequence).

You can leave the default settings as they are, or overwrite these with your own settings – The latter is only effective once you have clicked on „Selection of Pretesting Sequence determined by the user as shown above“.

Optionally, you can also add one or more of the following statistical methods to the pretest sequence:
Variance Analysis (ANOVA or Kruskal Wallis Test), **Jonkheere Terpstra Trend Test**, **Outlier Tests** (Dixon-Grubbs Test or Hampel Test). The result of these tests is not necessarily relevant for the selection of the final tests for NOEC determination, and therefore none of these tests have been set up as default tests. You can also add these processes in the automatic mode (this means without clicking on „selection determined by the user“. Read more about these methods in Chapter 4.7).

Now, we will take a look at the **final tests** (right side of the dialogue box). In the upper part of the box is a list of the available tests in ToxRat:

Williams Test,
Dunnet Test,
Bonferroni t.Test,
Bonferroni-Welch t-Test,
Step-down-Jonkheere-Terpstra Test,
Bonferroni-Median Test,
Bonferroni-U Test

Williams Test as the the most powerful test is preseleted. However, as long as the „automatic mode“ is activated (i.e. as long as „Selection of Final Test automatically by the program“ is selected at the bottom), this is only a “request” for the program – if the Williams Test will really be run, depends on the results of the pretest sequence (compare Figure 21). In the results, ToxRat explains why the decision for a specific test was taken. If you would like to set a specific final test for the NOEC determination – regardless of the pretest results – you must select „Selection of final test determined by user as shown above“. ToxRat will

then show the results of the pretests, but regardless of the results, will proceed with the user selected final test.
The default significance level for the final test is 5% (0.05), the default test direction for metric variables is “one sided smaller”. If desired, you can change both the significance level and the direction. The used values will be shown in the legend of the test results.

The following set up options for final tests is only relevant for experienced users:
“Include toxic or external standard”
Aside from controls, solvent controls, and treatments, you can also include positive controls or an external standard in the raw data. This can be found in the last column, and is normally not considered for the evaluation, unless you check this option. The data from this column will then be included in the multiple test for NOEC determination. This option should only be used in special cases by experienced users – but as a rule, is not advised, and therefore a warning is also shown when you select this check box. (Figure 25).

Figure 25: Warning when selecting „Include Toxic or External Standard“

4.4.2 NOEC Determination for Quantal Data

Note: You are not seeing double if some of the text in the following sections looks familiar to you. In order to present the information for metric and quantal data as stand-alone sections each, it is unavoidable to repeat some of the information.

Please use the following example files:
ToxRat Professional: Workbook OECD210
ToxRat Monitor: Workbook DIN EN ISO 10706
ToxRat Standard: Workbook „Testing a Quantal response 2 – Mortality replicated.xls“.

If you have opened a Biotest Workbook, then select, „Options – Hypothesis-Testing (NOEC)“. If you have opened a Generic Workbook, then use, „Find NOEC“ (compare Chapter 4.3). A window will open with all the procedures available which are shown in the test diagram for NOEC determination for quantal variables (Figure 26). This window is identical identical for both Biotest Workbooks and Generic Data Workbooks, with three exceptions:

1. The green variable check box, “Select variable for which adjustment applies“, can only be found in Biotest Workbooks, since only in these workbook several variables can be evaluated at the same time.
2. The check box, „Limit Testing / Two Sample Testing“ is only available in this menu in the Biotest Workbooks; in the Generic Workbooks you will find it (with identical content) under the menu item, „Find Limit Level“. We will discuss these methods in Chapter 4.5 , „Procedure for Determination of Limit Concentration“.
3. In the Biotest Workbooks, you will confirm your selections in this dialogue window by clicking on „OK“(below right). Subsequently, you will start the complete evaluation sequence with the RUN button in the evaluation window (compare Figure 12). In Generic Workbooks, you will confirm *and* start the selected settings with the RUN button (below right).

If several variables exist in a Workbook, then each variable will have its own standard settings. If you would like to change these settings, you must (and can) do this separately for each variable.

For users of ToxRat Professional and ToxRat Monitor: First select a quantal variable in the green variable check box. OECD 210: “Hatchability”; ISO 10706: „Mortality“.

You will now see the dialogue box „Testing Quantal Variables“ (Figure 26).
First, most importantly: The window closes at the bottom with a box, in which you determine if ToxRat should decide on which test sequence to use („automatically by the program“) or if your own settings should be used. („determined by the user as shown above“). Only if the latter is selected, your individual settings will be run!

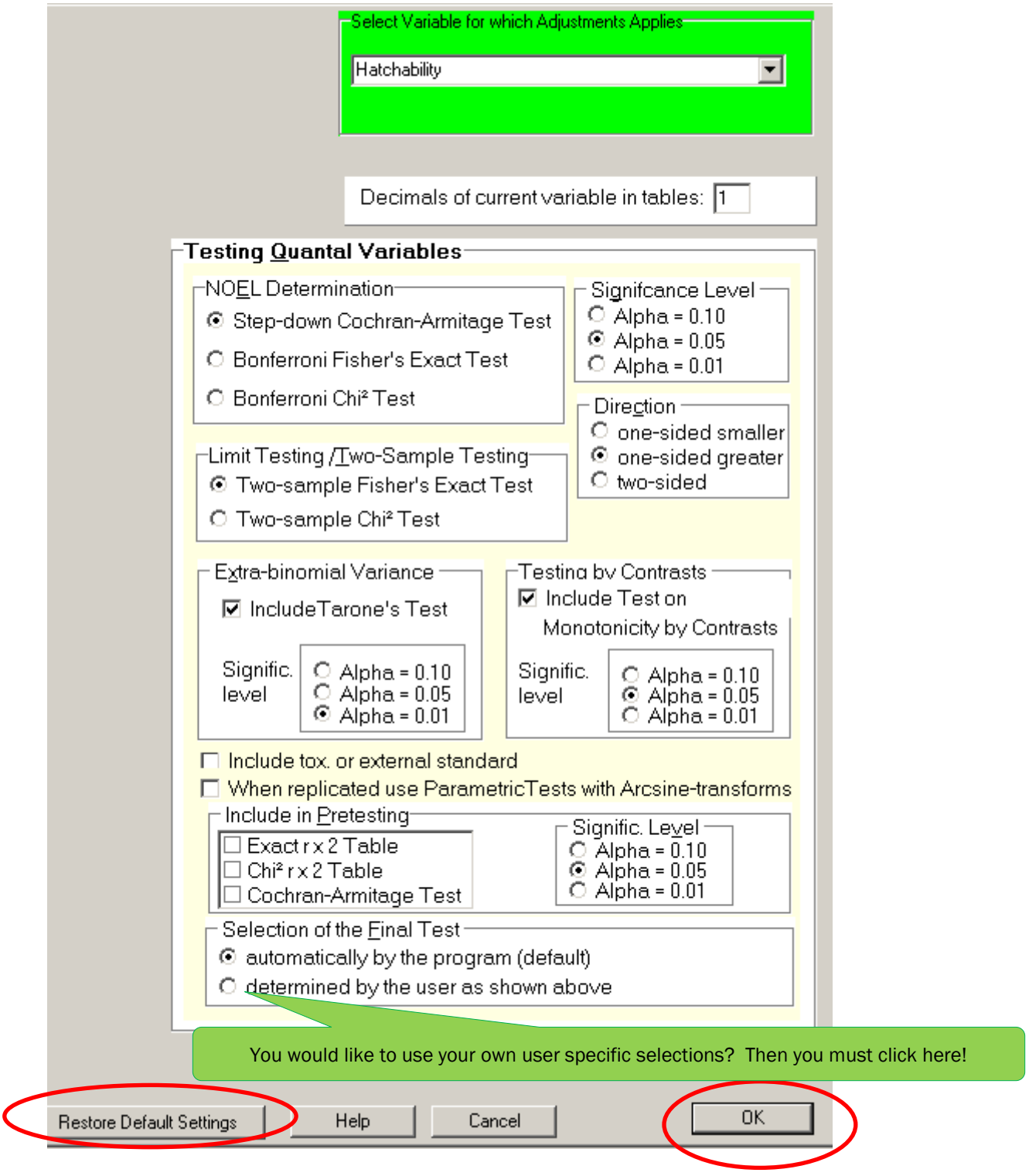


Figure 26: Dialogue window NOEC determination for quantal data

Standard settings are available for the mandatory NOEC determination tests for quantal data – these can be re-set at any time by clicking on „restore default settings“ (Figure 26, bottom left).

Your own settings will only be applied once you have clicked on the „determined by user as shown above“ button. Otherwise, ToxRat always uses its internal standard settings, regardless of what is selected in the NOEC options window.
Attention: this only applies to the mandatory testing, for which ToxRat recommends the default settings. Additional processes and settings („Include in pretesting“, „Include toxic or external standard“...) can also be added under the automatic mode.

Corresponding to the selection criteria for a suitable final test for NOEC determination (please see Figure 22), standard **pretests** have been activated, which check, if a monotone doses response relationship exists (Test on Monotonicity by Contrasts) and if an Extra Binomial Variance exists (Tarone Test, only possible if data is replicated). These tests are, in principle, run twosided, and therefore there is no possibility to change the test direction. For the Tarone Test, a significance level (error probability) of 1% is recommended, in order to reduce the risk of a false-positive result (and therewith an unnecessary weak final test).

There are three tests available for the **final test** for NOEC determination:

Step-down-Cochran-Armitage Test	If the there is an indication to extra-binomial variance in the Tarone Test, then the Cochran-Armitage-Test will be automatically run in the Rao-Scott variant.
Bonferroni-Fisher-Exact-Test Bonferroni-Chi2-Test	Both of these tests are principally based on the same procedure. For mathematical reasons the Fisher Test is not available any more as of a certain sample size. Should this test, nevertheless be selected, then a corresponding note will appear so that the evaluation can be repeated with the Chi2 Test.

Every test on NOEC determination is generally run with a significance level of 5% (0.05) and the preset test direction for quantal variables is „one sided greater “ (the percentage of “dead”, “not hatched”, or similar in treatments will be compared to control). If desired, you can set a different significance level or test direction. The selected significance level and test direction will be shown in the legend of the corresponding test results.

Optionally, you can also add one or more of the following statistical methods to the pretest sequence (“Include in pretesting”):
Variance Analysis by means of Exact rx2 Table Test or Chi2 Contingency Table (Chi2 rx2 Table). The exact test is more powerful, but may not work with very large sample sizes for mathematical reasons. In these cases, the Chi2 Contingency Test is available, that approximates the binomial distribution by the normal distribution.
With the **Cochran-Armitage-Test**, you can check if the observed effects follow a significant trend. The Tarone Test for extra binomial variance will be automatically run beforehand. If this is significant, the Cochran Armitage Test will be run in the Rao-Scott Variant for data showing extra binomial variance.

The result of these tests is not relevant for the selection of the final test for NOEC determination, and therefore none of them are pre-selected. You can also add these processes in the automatic mode (this means without having to click on „selection determined by the user“).

The option **„Include toxic or external standard“** is meant for experienced users for specific special cases only and is otherwise not recommended. Please read the corresponding section on metric variables for more on this (Figure 25).

Transformation

Finally, if the quantal data is replicated, it can be transformed and then evaluated with the parametric tests for metric data (compare Figure 22). To run a data transformation, please place a check mark in the options window „Testing Quantal Variables“ at the option „When replicated use parametric Tests with Arcsin-Transformation”. An additional window will then open: „Testing Metric or Transformed Variables“, which you already know from the metric variables (Figure 27). ToxRat will now transform the quantal data in the background (see also Chapter 4.2.) and then perform the procedures of the parametric test process determine an NOEC – either using default settings or using your individual user settings in the box “Testing Metric or Transformed Variables”. For details please refer to Chapter 4.4.1..

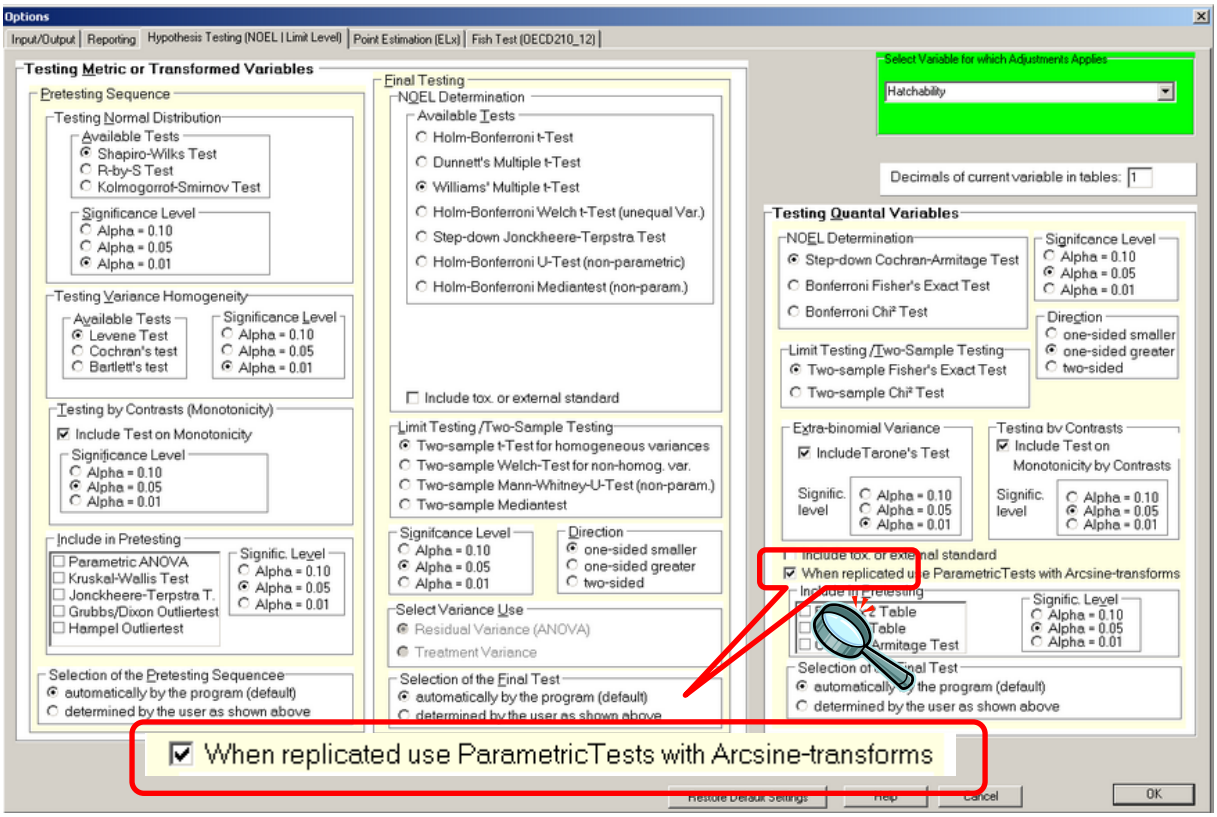


Figure 27: NOEC Determination for transformed quantal variables

By transforming quantal data, it now meets the characteristics of metric data and the numerical values are reversed – this means the higher the measured mortality of the untransformed data record are, the lower the numerical value of the transformed data record will be. Therefore, ToxRat will also automatically change the test direction.

4.5 Procedure for determining limit concentrations / two-sample tests

Two-sample comparisons are used

- if only one treatment has been tested, e.g. for determining a limit concentration
- where several treatments are to be independently compared with the control

Try the following sample files:
ToxRat Professional: Workbook OECD210
ToxRat Monitor: Workbook DIN EN ISO 10706
ToxRat Standard: Workbook „Testing a Metric response 3 at several Intervals.xls“.

For two-sample testing, the same conditions and preliminary considerations as for NOEC testing generally apply (preliminary tests with standard sequence and variance homogeneity, level of significance, direction). The only difference is that two-sample and not multi-sample tests will be conducted.

A **Generic Workbook** allows the direct individual selection of all available two-sample tests – the procedure will be explained in detail in chapter 4.7.
Alternatively, you can leave the selection of an appropriate test to ToxRat by selecting the menu item "Find Limit" (generic workbooks) or "Options – Hypothesis Testing (NOEC) / Limit Level" (Biotest Workbook). Depending on the type of variable selected, a dialogue for quantal or metric responses will open (Figure 28 and Figure 29). The complete test sequence including the final two-sample test (Generic Workbook) is started by clicking RUN.

In a **Biotest Workbook**, you either conform the default settings or your own settings by clicking “OK”, and then start the evaluation process by clicking RUN in the Main Menu bar.

It would be a good idea to go ahead and read the sections on NOEC testing (chapter 4.4) now – the backgrounds and operating steps described there also apply for the two-sample testing procedure described above.

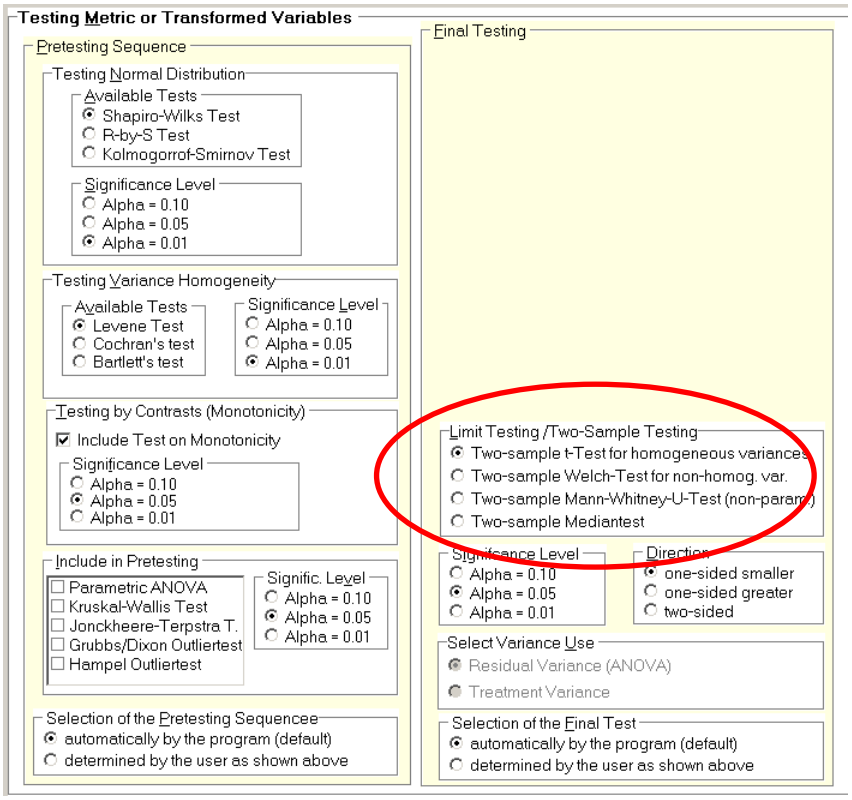


Figure 28: Dialogue window for the selection of two-sample tests for a metric response

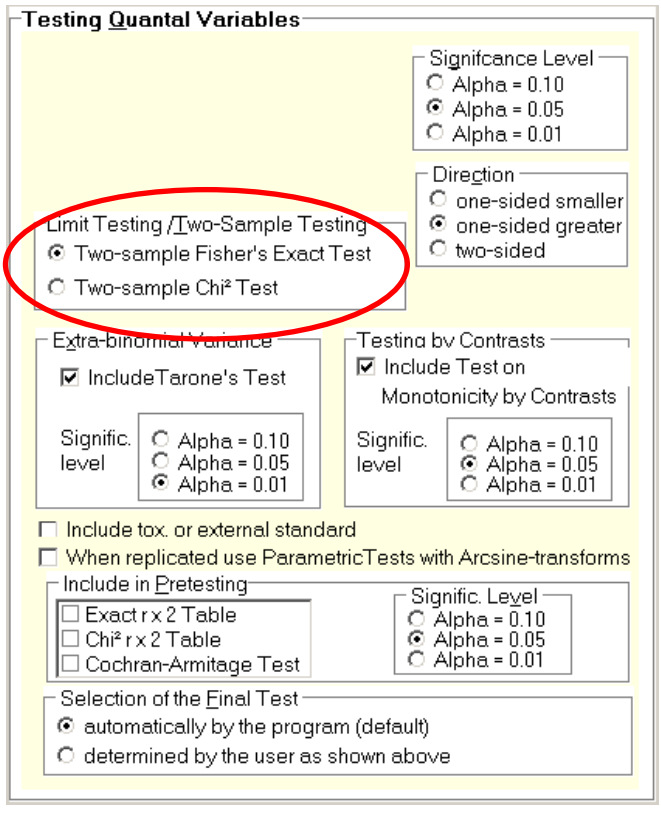


Figure 29: Dialogue window for the selection of two-sample tests for a quantal response

4.6 Determining Effect Level, ELx

Try the following sample files:
ToxRat Professional: Workbook OECD210
ToxRat Monitor: Workbook DIN EN ISO 10706
ToxRat Standard: Workbook „Testing a Metric response 3 at several Intervals.xls“.
Workbook „Testing a Quantal response 2 – Mortality replicated.xls“.

Preliminary Remark:
According to recent guideline requirements, the new ToxRat default settings for metric data are „non-linear regression“ rather than linear regression. If you still prefer linear regression, please change settings manually using the dialogue window for effect level calculation (see below).
The effect level (ELx) will be concentrations (ECx), doses (EDx), or rates (ERx), depending on the type of application. You can select the appropriate abbreviation for the result tables via the Options menu -> Input-Output (Figure 30: Selection options for effect level designations (Menu “Options” / “Input-output”). In the generic workbooks, the default setting is “ECx” for “Concentrations”. In the biotest workbooks, the designation matching the application type specified in the guideline is preset.
For the purposes of this manual, we will be using either the general designation “Level” or - representative of all application types - the designation “Concentration”.

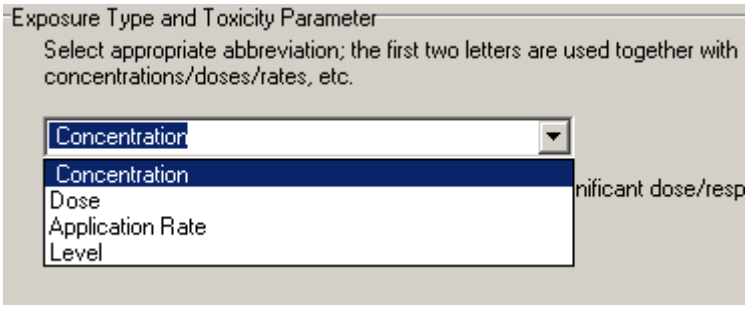


Figure 30: Selection options for effect level designations (Menu “Options” / “Input-output”)

Where do I find the dialogue window for Effect Level Calculation?
With the Biotest Workbook open, select “Options” / “Point Estimation (ELx). Available methods (Linear Regression / Non-linear Regression / Interpolation) will be displayed depending on the variable set in the green box at the top right.
Click the grey button to activate the method you want. The selected method will now be highlighted in green. Settings can only be changed after the selection window for a method has been activated.
With the Generic Workbook open, select the menu option “Find Effect Level”. A pulldown menu will open for metric variables, offering a choice of “Linear Regression” or “Non-Linear Regression”. For quantal variables, select either “Linear Regression” or “Interpolation”.

Note for users of the previous version ToxRat 2.10.05:
In generic workbooks, you will find the selection dialogue for the effect level calculation directly via the menu item “Find Effect Level”. The previously more roundabout way via “Options” is now obsolete! Click RUN in the selection dialogue to initiate the calculation.

Specifying the Effect Levels to be calculated, Extrapolation factor

Interpolation methods as a rule only permit the calculation of EL50 responses; for linear and non-linear regressions, on the other hand, you can choose up to six freely selectable effect levels for calculation, including confidence intervals (Figure 31). If you want more than 6 effect levels to be shown, please see chapter 6.5.3.
The so-called "Extrapolation Limiter" plays an important role in the process. The extrapolation limiter is a software setting that will ensure that – in accordance with the OECD Statistic Document 2006 requirements[2] - only ELx responses within the examined concentration range will be returned, i.e. only those that are directly supported by test data. Where an ELx response can be determined only by way of extrapolation, then ToxRat will initially respond with “n.d.” (not determined).
A response with extrapolated ELx data can, however, be forced by replacing the default value (“-1” = no extrapolation) in the menu “Point Estimation to Calculate” with a so-called extrapolation factor (Figure 31). We recommend carrying out an initial evaluation with the default value “-1” to prevent accidental extrapolation.

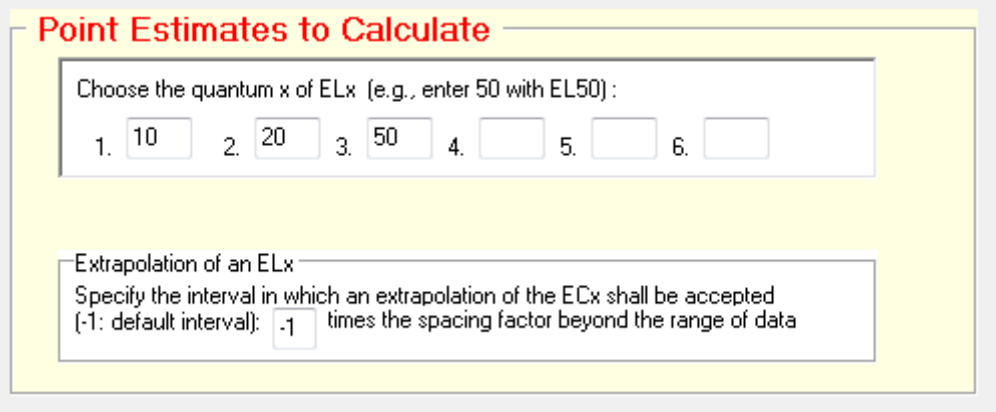


Figure 31: Dialogue for the selection of the appropriate Effect Level for calculation and for configuring the "Extrapolation Limiter"

The following sections offer information about setting options for linear and non-linear regression, and for interpolation methods. The validation document contains details about the relevant mathematical approaches; it is meant to offer you some basic decision tools for correct method application.

[2] Current Approaches in the Statistical Analysis of Ecotoxicity data: A Guidance to Application, OECD Series on Testing and Assessment, Number 54, ENV/JM/Mono (2006) 18

4.6.1 Linear Regression

The objective of linear regression is to create a linear dependency for a non-linear relationship between two variables by transforming either one or both variables. In effect, linear regression is as a general rule based on transformed data, rather than on original data.

Important characteristics of linear regression are:

- Normality and variance homogeneity are required / approximated by weighting factors
- Based on transformed data (see above)
- Normalization of metric data required (i.e. data are related to control (percent inhibition); no need for normalization for quantal data, since these are always related to individual starting values (i.e. pertentage of dead of a certain number of introduced)
- Only 2-parameter-functions available (for details see below)
- Minimum and maximum inhibition 0% and 100% (for details see below)
- Three functions available: Probit, Logit, Weibull (for details see below)
- Optimization algorithms: maximum-likelihood regression (Standard); simple linear regression, weighted linear regression (for details see below)
- Calculation of confidence limits using either Fieller´s theorem (Standard), Normal approximation or Bootstrapping. Option for correcton of variance (metric data) or correction for heterogeneity (quantal data). For details see below
-

Before you continue: Please make sure to read first the general information about menu navigation and general settings (chapter 4.6).

Once again: Leave everything as it is, and carry out a first evaluation using the default settings.

In the following, we will explain some of the default settings and available alternatives. Depending on the data set, you then may vary the settings for finding a linear regression that will best describe the functional relationship between variable and concentration (Figure 32, Figure 33).

Data Adjustment for Normal Distribution

In linear regression min. and max. inhibition values are 0% and 100%. Inhibition values greater than 100% are therefore automatically set to 100, and values less than zero (i.e. stimulations) are set to zero.

Previously, the algorithms simply could not process values of 0% and 100%, and these were replaced with e.g. 0,1% and 99.9%. Although there is no longer a mathematical necessity for it, you can activate the so-called “Data Adjustment” here to reproduce previous evaluations.

Available Functions

ToxRat offers three different two-parameter functions for calculating the inhibition value by way of linear regression. Each one of these presumes a specific type of dose response relationship.

- Probit, based on cumulative normal distribution
- Logit, based on the logistics function
- Weibull, based on the exponential function

Next to these and in the same dialogue, you will find the option

- Linear (straight line)

This function is used not a method for inhibition value calculation.

The option "Linear (straight line)" effects a simple linear regression using metric original data, i.e. without transformation. Wirth quantal data the function cannot be selected. This procedure is a requirement for some validation steps in ToxRat 3.0 or higher (see validation document). Since it is not a method for effect level calculation, no EC-values are calculated. You are, however, free to use this function for calculating simple linear regressions in ToxRat.

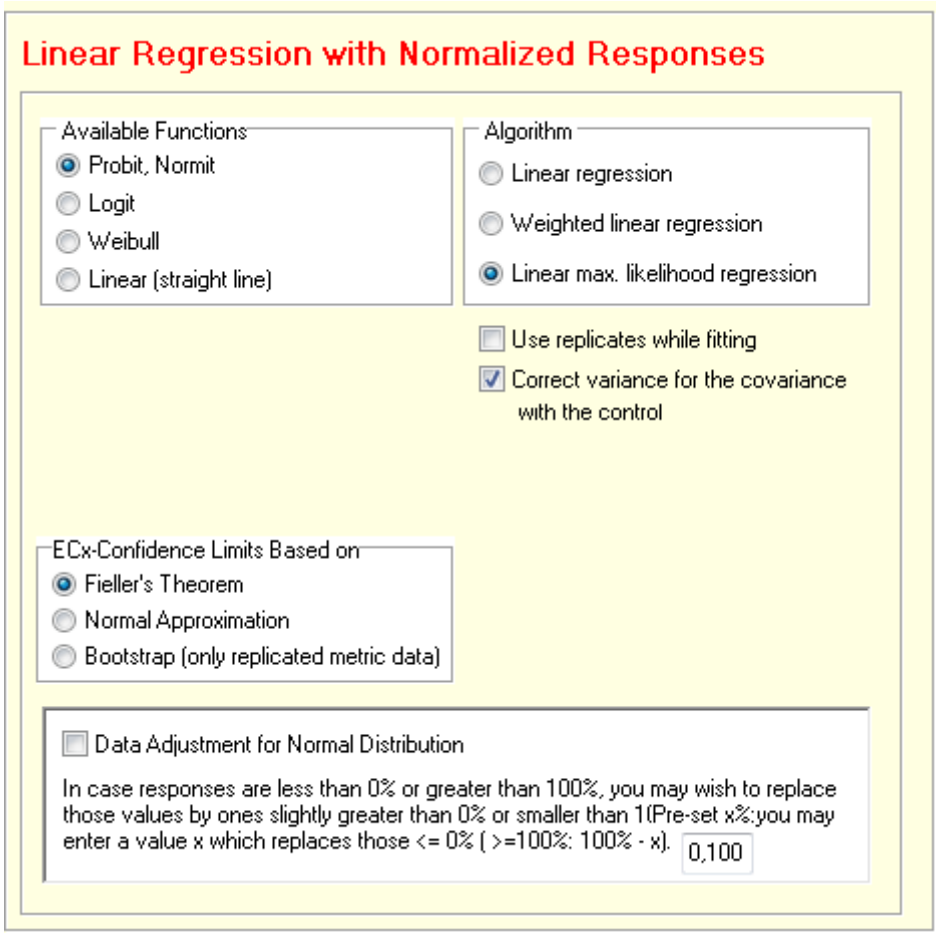


Figure 32: Method selection dialogue for linear regressions using metric variables. Software default settings.

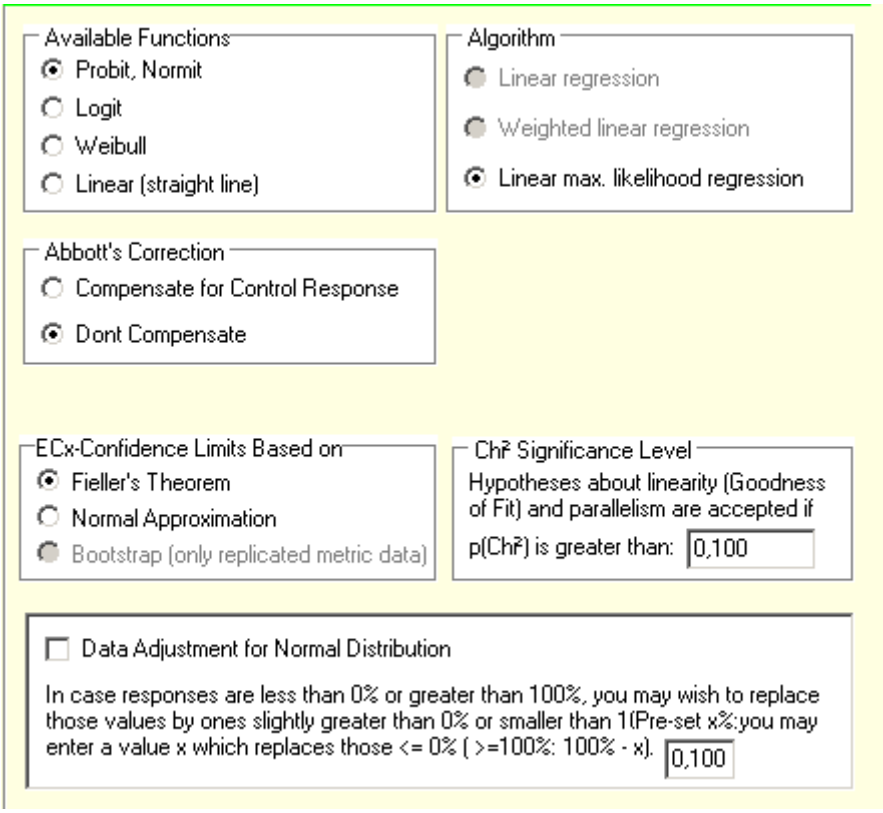


Figure 33: Method selection dialogue for linear regressions using quantal variables. Software default settings.

You may have to experiment a little to find out, which function offers the best fit (i.e. "Goodness of Fit"). The most common function is the Pürobit function, that´s why it is preset by the program. Fit quality is - aside from a purely visual assessment - evaluated in accordance with so called “Quality Criteria”, which ToxRat provides automatically. ToxRat furthermore verifies, whether or not a significant dose-effect relationship actually exists. For more information about these decision-making aids, please read chapter 6.3.3.
In the area around EC50, the results of Probit, Logit, and Weibull functions are mostly the same, but there will be differences in the end ranges of the function, around EC10 and EC90.

The Probit analysis was developed originally for quantal data. When it is used for metric responses, then ToxRat will take into account specific weighting factors from literature. The same applies for the Logit and Weibull functions. Please read the validation document for more information on the topic.

Algorithm

During the actual fitting procedure, i.e. the adaptation of a function to the data, the two parameters a and b of the function are determined by way of a specific calculation process. Available for selection are

- Linear Regression
- Weighted Linear Regression
- Linear Max. Likelihood Regression

The default setting for Maximum Likelihood will deliver the most exact results. As a rule, you will not be needing any other settings.

Fit with Mean Values and Replicates

For metric, replicated results, you can decide whether to carry out the fitting process with mean data or the individual replicates (
Figure 32, "Use replicates while fitting"). Unless stated otherwise in the Test Guideline document, this check box will not be selected, i.e. mean values will be used by default.

Should a fit based on the calculation of mean values not return a significant dosis-effect relationship, then the use of replicates may help to achieve significance!

Confidence Limits calculations ("ECx Confidence Limits Based on")

Depending on the data available, the selected function can be calculated more or less confident. So-called “Confidence Limits” are one way to quantify the uncertainty of a derived dose-effect function, and therefore also for the validity of the resulting ECx values. Confidence intervals define the range in which the function (or the ECx values) can be found with 95% probability. The more certainty can be applied for the function, i.e. the better it is supported with data, the narrower the confidence limits. In effect, confidence limits are a means for quantifying the validity of an ECx value. Prerequisite for the calculation of confidence limits with ToxRat is that there is a dose response relationship at all. Where that is not the case, ToxRat will as a rule not output any confidence limits, and will offer instead "n.d" ("not determined"). For details please see chapter 6.3.3.

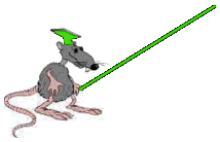
ToxRat offers the following processes for confidence limit calculations:

- Fieller´s Theorem (software default setting)
- Normal Approximation
- Bootstrap Process (exclusively for metric replicated data)

The Fieller default process (as well as the Normal Approximation) does not consider the control variance due to the normalisation (control value division). As a consequence, the confidence limits might be too close. For this reason, ToxRat enables to consider control varianceThe Bootstrap process offers an alternative, which includes the control variance:

Variance correction for confidence range calculations ("Correct variance with the covariance of the control", available exclusively for metric data)
An option to include the control variance also if Fieller´s theorem is selected, is a special correction factor as described in Appendix 5 of the Test Guideline OECD 201 (2006, Appendix corrected 2011) for non-linear regression. This will be a default option in ToxRat 3.0 for confidence range calculations in linear regression.

Variance correction for confidence range calculations in metric data ("Correct variance with the covariance of the control") can be deactivated to allow you to reproduce results from earlier ToxRat versions.



The confidence range calculations will be conducted on the basis of the mean values if you have selected using the mean values for your fit. In combination with variance correction, this can result in quite broad confidence ranges, depending on the data used. You could therefore alternatively try using replicates (select "use replicates while fitting"), as this may very well help to decrease your confidence ranges!

Heterogeneity Correction (only for quantal data)
One measure for the level of congruity between data and fit (goodness of fit) is the so-called "Chi^2 level of significance" p(Chi^2). Possible values for p(Chi^2) are between 0 and 1, whereby "1" means max. congruity (see also chapter 6.3.3.). Where a great heterogeneity exists, i.e. p(Chi2) falls below a specified critical limit, the confidence interval will be extended accordingly. This process is referred to as "heterogeneity correction".
The preset limit value for p(Chi2), at which heterogeneity correction is triggered, is 0.1%. This value can be adjusted here.

Abbott Correction (only for quantal data)
When the relevant check box is selected, then all mortalities (i.e. inhibitions) are reduced by the control mortality value. The mathematical process, on which this is based, is called "Abbott Correction". Regression calculations are then executed on the basis of the corrected inhibition values.

4.6.2 Non-Linear Regression

The objective of non-linear regression is to describe the relationship between two variables using an appropriate function. Other than in linear regression, data is not transformed, i.e. the original measurement values are used in the calculation.
Important characteristics are:

- Normal distribution and variance homogeneity are not a prerequisite
- Based on original data (see above and example in Figure 85)
- No normalisation of metric data required (normalisation = referencing of the measurement values to "Control" → % Inhibition); Exception: Two-parameter functions
- three available function families (cumulative distribution functions, CDF): based on normal distribution (Normal CDF), based on logistic function (Logistic CDF), based on Weibull function (Weibull CDF); see below for more information;
- In ToxRat, 2, 3, and 4-parameter functions are possible for each function family; depending on the number of parameters, the min. and max. values to be included in the regression will differ. See below for more information.
- The data can be included in the regression as unweighted or weighted, and there are several weighting types available. See below for more information.
- Available optimisation algorithms for the function in ToxRat: Levenberg Marquardt (default setting), Downhill-Simplex
- Confidence limit determination by way of Monte Carlo simulation (where Levenberg Marquardt is selected as optimisation algorithm; default), or by way of bootstrapping (where Downhill-Simplex is selected as optimisation method); see below for more information.

Especially for metric data the non-linear regression is an important alternative to linear regression, as it can do without data normalisation. Since for quantal data normalization is not required at all, for these data linear regression will generally be the right choice with .
ToxRat 3.0 offers methods for the non-linear regression exclusively for metric data. Future versions of the software will also include non-linear regression for quantal data.

Please read the general information about menu navigation and general settings (chapter 4.6, page 49) before carrying out a non-linear regression with ToxRat.

Non-linear regression is a lot more complex than its linear counterpart, which means that there are a lot more factors and setting options, which can all influence the outcome (see Figure 34 and section 6.3.4). In particular, the type of weighting (see below: "Further Options – Regression Type") will have a huge impact on the result of the regression, but is very difficult to standardise from a programming point of view. Nevertheless ToxRat does offer some presets here, i.e. you can use the default settings to carry out a regression. However, depending on the data you have available, you may have to change some settings manually and try out some of the variations to get an optimised result.
You can use the so-called "Goodness of Fit" criteria (which ToxRat outputs automatically) in addition to your own visual assessment to see, which settings offer the best fit for your data. ToxRat furthermore verifies, whether or not a significant dose-effect relationship actually exists. You will find out more about results assessment in chapter 6.3.4.

Figure 34 offers an overview of the factors you can try out in non-linear regressions. The boxes with a bold frame are the default software settings: 3 parameter Normal CDF with Levenberg-Marquardt algorithm, non weighting, and confidence intervals by way of Monte Carlo simulation.
In this chapter we will look at the menus for non-linear regression, and explain the various setting options.

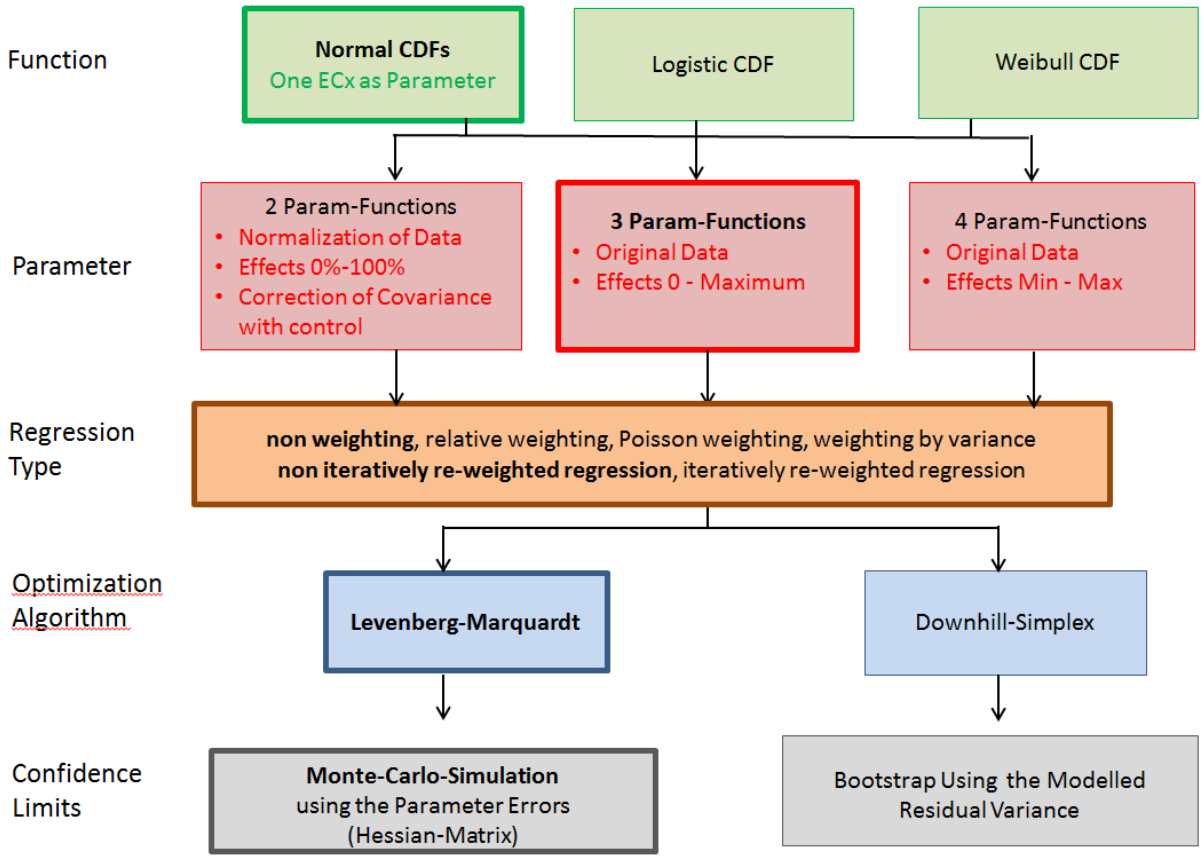
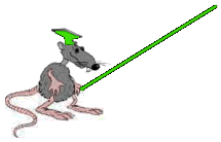


Figure 34: Overview of the processes in non-linear regression for metric data in ToxRat. Bold print: Software default settings



Rule of thumb: Always select the most simple model that will give reasonable results.

Select the type of function in the main dialog for non-linear regression (Figure 35).

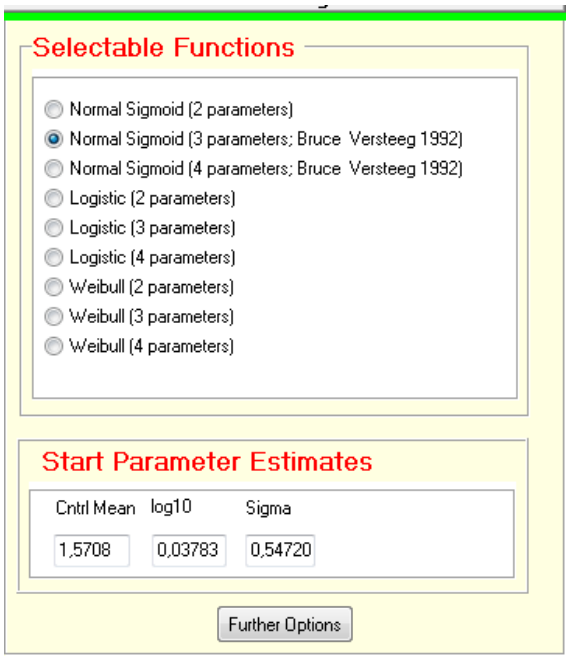


Figure 35: Selection dialog for available non-linear regression methods; default settings: 3 parameter Normal CDF

Available Functions and Start Parameters

Choose between three function families (cumulative distribution function = CDF):
Normal CDF (based on normal distribution)
Logistic CDF (based on logical function)
Weibull CDF (based on Weibull function)

ToxRat automatically provides start parameters for the optimisation algorithm for each selected function. These are not fixed, and will be estimated in the background for each data set individually during the read process. For Generic Workbooks Metric responses, the start parameters are shown (see Figure 35, box "Start Parameter Estimates"); with Biotest Workbooks, this step in performed unvisible in the background. Unsuitable start parameters could mean that the regression terminates without a result. That is why only advanced users should make amendments to start parameters! If you did go ahead and changed something, and now want to undo the change: ToxRat will define the start parameters automatically with every "refresh".

One of the selected EC values will as a rule be set as a function parameter for the Normal CDFs. In ToxRat, this will automatically be the first of the selected EC values (see Figure 31, box "Point Estimates to calculate").

If you don't want the smallest of the selected EC values to be set as start parameter, then you can simply change the input sequence individually

Number of Function Parameters

Each of the available function families can be fitted to the data with either 2, 3, or 4 parameters. It depends on the number of function parameters, which effect range or data range can be included in the fit:
2 parameters: 0% to 100%
3 parameters: 0 to max.; max. depending on original scale
4 parameters: Min. to max.; min. and max. depending on original scale (i.e. an EC is relative, as it is related to the span between max. and min. (span = 100% effect, min. = max. achievable effect)
In 2 parameter functions, a data normalisation is required - just like in linear regression.

The default setting here is 3 parameter Normal CDF according to Bruce-Versteeg, which is recommended in many guidelines.

The more complex the function you want to use for describing your data, the more information will be required to determine the result. Rule of thumb: You can only fit as many parameters as there are test concentrations (without control).

ECx Values and Extrapolation Factor

You can specify up to six EC values in the box "Point Estimates to Calculate", which are to be output with their confidence intervals. More on this topic, and specifically on the extrapolation factor can be found in the general information about regression in chapter 4.6.
If you have selected a function from the Normal CDF family, then *the first EC value entered here will automatically be one of the function parameters.* If you don't want the smallest of the selected EC values to be set as start parameter, then you can simply change their input sequence. Please note: the confidence limits of the ECx which is set as parameter, will be calculated directly from the standard error of this parameter and are thus more precise (i.e. usually narrower). Other confidence limits are calulated by Monte Carlo simulation. I.e. setting an ECx as parameter means minimizing the width of CI exactly for this ECx.

Further Options

You can start your non-linear regression, once you have selected the output EC values and the function to be used – ToxRat will then use default values for all other setting options. If you are not satisfied with the result (see "Goodness of Fit Criteria", chapter 6.3.4), please try additional setting options via the button “Further Options” (Figure 36).

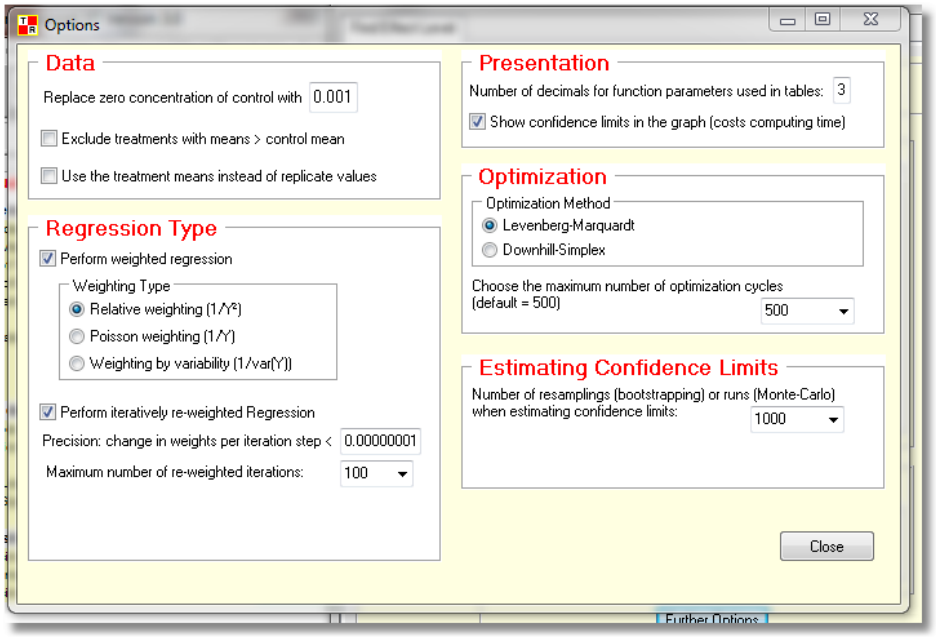


Figure 36: Special setting options ("Further Options") for non-linear regression; software default settings; Important: "Perform weighted regression" and "Perform iteratively re-weighted Regression" is not activated by default; it is selected for this screenshot for reasons of completeness, as other setting options ("Precisions change", "Maximum Number") would otherwise not be visible.

Data

Replace zero concentration of control with xxx

Here you can specify the value you want to input for the control concentration. The default value is "0.001". **Important:** The repacing value must be so small that a further reduction has no influence on the determined ECx values. A simple check, whether the control replacing value is appropriate, can be done visually: *the control data need to be located in the plateau area of the regression curve – i.e. in the area beyond visible confidence limits* (see e.g. Fig. 85, p 101).

You can either decrease the value for the control concentration, or, alternatively, you can keep the default value of 0,001 and enter the concentrations in a different unit of measure as well (e.g. milligram instead of gram).

Exclude treatments with means > control

This option is relevant if some of the treatments have returned stimulations, i.e. the mean value of the treatment is greater than the control mean. The following options are available to deal with these treatments:

- Fitting the function including all measurement values, i.e. include the data from treatments with stimulations in the function parameters. Important: This is not a hormesis model (this option is not yet available in ToxRat 3.0); instead, the selected function will remain unchanged (Normal CDF, Logistic CDF or Weibull CDF) – only the top part of the curve will be slightly shifted in the coordinate system.
  This option will be performed if the check box "Exclude treatments..." is not activated (default setting).
- Leaving out these treatments for the calculation of function parameters. This option is comparable to the practise in linear regression, where stimulations are replaced by zero % inhibition. This option will be performed if the check box "Exclude treatments..." is activated.

Use the treatment means instead of the replicate values

Non-linear regression is generally carried out on the basis of individual replicate values. Here you have the option to switch to mean values. This might be helpful if you are trying to reproduce results from publications, or in case of specific data situations, where a regression will work better on the basis of mean values. This method also makes sense if there is no raw data available, and mean values were entered directly in the data input sheets, as for means a variance correction for determining confidence intervals is carried out with a different formula than for replicates (only relevant for 2 parameter CDFs).

Type and Weighting of Regression

Non-linear regression can be done with or without weighting. The weighting can be done on two levels:

1. "weighted regression"
   The measured data values are weighted in order to calculate the function values. Specific weighting types are as follows:
   - Relative weighting ($1/Y^2$): the lower the absolute numerical value, the higher the weight --> might be helpful if there are only few data existing with high inhibitions, e.g. because of lower number of replicates. Relative weighting gives greater condideration to these data.
   - Poisson weighting (1/Y): might be helpful with data showing a poisson distribution, e.g. count data.
   - Weighting by variance (1/var(Y)): the higher the variability, the lower the weight. Helps to reduce the disturbing impact of treatments showing high variability

The weighting function is not activated by default, as weighting is optional.

2. "iteratively reweighted regression"
   The resulting predicted values are reweighted step by step, and the calculation using the changed weighting factors is repeated until the predicted values no longer change, or until the cancel criterion (max. number of iterations) is reached. Not activated by default.

The success or failure of the regression may depend on the type of weighting set under "Regression Type".
Weighting is not a must! Therefore ToxRat at first performs a regression without any weighting. Should the regression using default values not return a satisfactory result, then you should definitely try out different types of weighting. The higher the span between the data, the greater the impact of weighting.

Once you have found a weighting that fits, you can probably still improve your results using the iterative reweighted regression.

Presentation

The confidence limit calculation is - specifically in the Bootstrap method - very calculation-intensive and can therefore take quite some time to complete (depending on the capacity of your computer). A mapping of the entire confidence interval in a graph is therefore optional.
Here you can modify the number of decimal digits displayed for the function parameters in the result tables, e.g. if one of the resulting parameters is so small that three decimal digits (default) won't be enough.

Optimisation Algorithms

ToxRat offers two separate algorithms with which to calculate function parameters:
The method according to Levenberg-Marquardt (default), and the Downhill-Simplex algorithm. The number of optimisation cycles to be performed with the selected algorithm can also be specified. The default setting here is 500 cycles.
The Levenberg-Marquardt method has the advantage that standard errors are defined for each function parameter, which allows direct confidence limit calculations. Let's say you only need one EC value and a Normal CDF was selected (i.e. the EC value is also a parameter). Then the Levenberg-Marquardt algorithm will automatically return the standard error from which the confidence interval for this EC-value can be calculated directly. Confidence limits for additional EC values or for all predicted values (where a mapping of the confidence limits in a graph is desirable) will be calculated using the Monte Carlo simulation, see below. Standard errors also allow carrying out a t-test for significance for each function parameter. That is why ToxRat. comes with Levenberg-Marquardt as default setting. Nevertheless, if the regression using the Levenberg-Marquardt algorithm fails, Downhill-Simplex from experience might be successful and therefore should be tried instead (but see below: minimum number of replicates required).

Estimating Confidence Limits

Confidence limits cannot be calculated directly (exception: where standard errors are known, see above) - they must be determined using simulation methods.
The Levenberg-Marquardt algorithm will use the Monte Carlo simulation, the Downhill-Simplex algorithm will use Bootstrap procedure here. The relevant number of cycles (Monte Carlo) or samples taken (bootstrapping) can be set here - experience has shown that a value of 1000 will return usable results (default setting). Bootstrapping is, however, significantly more calculation-intensive, i.e. the evaluation run will take markedly more time. Please note, using Bootstrapping to calculate confidence limits requires at least 6-7 replicates per tzreatm,ent, otherwise the confidence range might be underestimated.

4.6.3 Interpolation

Before you continue: Please make sure to read the general information about menu navigation and general settings for effect level calculation (chapter 4.6, page 49).

Interpolation methods for inhibition value calculation are based on the binomial distribution, and are therefore available only for quantal values. These methods offer an option of calculating inhibition values and confidence limits even if the available data does not suffice for a regression procedure. Interpolation returns only EC50 values, which means you will not be able to set any other effect levels. EC50 values are always determined by way of linear interpolation using logarithmized concentrations. The number of concentrations included in the interpolation and calculation of confidence limits will vary depending on the method used.
Figure 37 shows the available methods and optional settings. The available interpolation methods are grouped in accordance with their reliability and accuracy. You should therefore follow the priorities shown in the selection dialoge – provided the data is sufficient.
You can either keep the default settings or make your own selection – then you start the evaluation process by clicking RUN (at the bottom right of the Find Effect Level dialog).

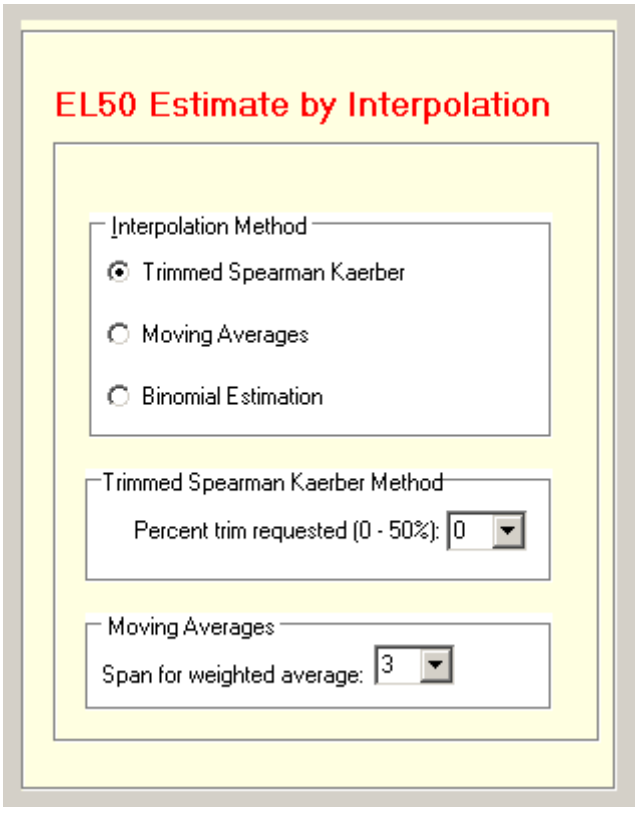


Figure 37: Selection dialog for the inhibition value calculation of quantal variables using interpolation methods

Spearman Kärber

The Spearman-Kärber method presumes a constant spacing factor and monotonous dose-response relationship. The observed dependency will be smoothed as needed. If the data record does not contain any processing with 0% or 100% inhibition, then the next lower or next higher concentration will be added (while maintaining the spacing factor), and will be presumed with 0% or 100% inhibition. That means that in principle an EC50 value can be determined even if the data does not include a 50% inhibition value. The value of that operation should be examined critically on a case by case basis, also taking into account that an extrapolation of EC values from within the data range is generally not recommended[3].

The EC50 value is estimated as a moving average across all concentrations unless concentrations were trimmed ("trim", see below). Confidence interval calculations are based on a variance estimate. Only one test approach with 0% or 100% effect each will be included in the confidence limit calculation, where several test approaches with 0% or 100% exist.

In the Trimmed Spearman-Kärber version, inhibitions up to / from a certain value will be excluded for the calculation of EC50 or the confidence interval. You can specify the cut-off or “trim” for inhibitions in the dialog “Percent trim requested”. The default setting 0% represents the original Spearman-Kärber method without trim. 25% means that only inhibitions between 25% and 75% will be included.

Moving Average

The Moving Average process calculates a moving average across all inhibitions in a specified number of treatments. The number of treatments to be included in the interpolation is specified via the so-called “Span”. Have a try with the preset number of "3" – if there is no result, then ToxRat will prompt you to edit the Span value.
Generally, an EC50 calculation will only be possible if the measured inhibition values include 50%.
The calculation of confidence limits is — just like in the Spearman-Kärber process — based on a variance estimate using all measured inhibition values, which means that even those values are included that are outside the selected Span.

Binomial Estimates

The binomial process calculates the EC value by way of a linear interpolation between the two measured inhibition values that include 50%.
The confidence limits are estimated from the cumulative binomial distribution by setting those test concentrations as upper and lower limits for the confidence limit, whose cumulative binomial probabilities include 95% of the data. Depending on the data at hand, this can be a very broad range of concentrations. The lower or upper limit of the confidence interval cannot be determined if the lowest measured effect is significantly greater than 0%, or if the highest effect is markedly below 100%, because the cumulative probabilities will then not cover the 95% range.

[3] Current Approaches in the Statistical Analysis of Ecotoxicity data: A Guidance to Application, OECD Series on Testing and Assessment, Number 54, ENV/JM/Mono (2006) 18

4.7 Manual selection of individual statistical procedures

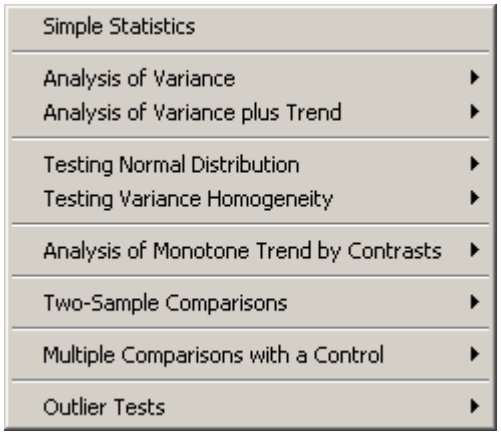
Please use the following sample files:
ToxRat Standard: Workbook „Testing a Metric response 3 at several Intervals.xls“.
Workbook „Testing a Quantal response 1 – Mortality.xls“.

Use the menu item “Statistical Procedures” in the generic workbooks (Figure 38) if you would like to select and try some specific procedures.

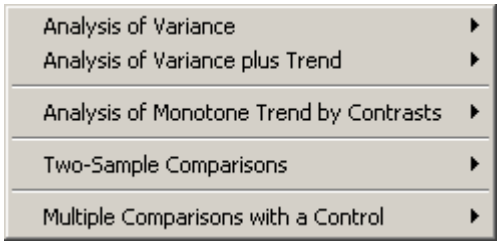
Figure 38: Menu item "Statistical Procedures" in the open generic workbook

You have already learned some basic information about this menu item in Chapter 4.3.2 – in the interest of completeness, some of the information is presented here once again (in more detail). You will then get to know the available methods individually.

The headers in the pull-down menu “Statistical Procedures” and the content of each of the submenus are all context-specific, i.e. ToxRat will display only those methods applicable for the current variable type and data set, depending on whether you have opened a workbook for quantal or metric data (Figure 39). Of all the theoretically applicable tests, ToxRat will then execute your selection – irrespective of whether the relevant requirements (e.g. normality or variance homogeneity, see Chapter 4.4) are met. Alternatively, you might select the menu items “Find No Observed Effect Threshold” or “Find Limit Level” if you would prefer the software’s support for your test selection.



Metric data or replicated quantal data following transformation



Quantal Data

Figure 39: Content of the pull-down menu “Statistical Procedures” for a) metric or transformed quantal data, and b) quantal data.

Quantal data — provided it is replicated — can as a rule be transformed for subsequent evaluation using parametric tests for metric data. That is why the menu "Statistical Procedures" also includes methods for metric data for replicated quantal data. ToxRat will carry out the required data transformation automatically if one of these methods is selected. The result tables will contain a reference to the transformation. Figure 41 to Figure 48 show the original methods for quantal and metric data.
Whatever the method selected, ToxRat will always generate a diagram of "Measurand against Time".

A dialog will open if you have selected a statistical method with setting options, where you can specify important framework conditions by selecting a radio button (Figure 40).

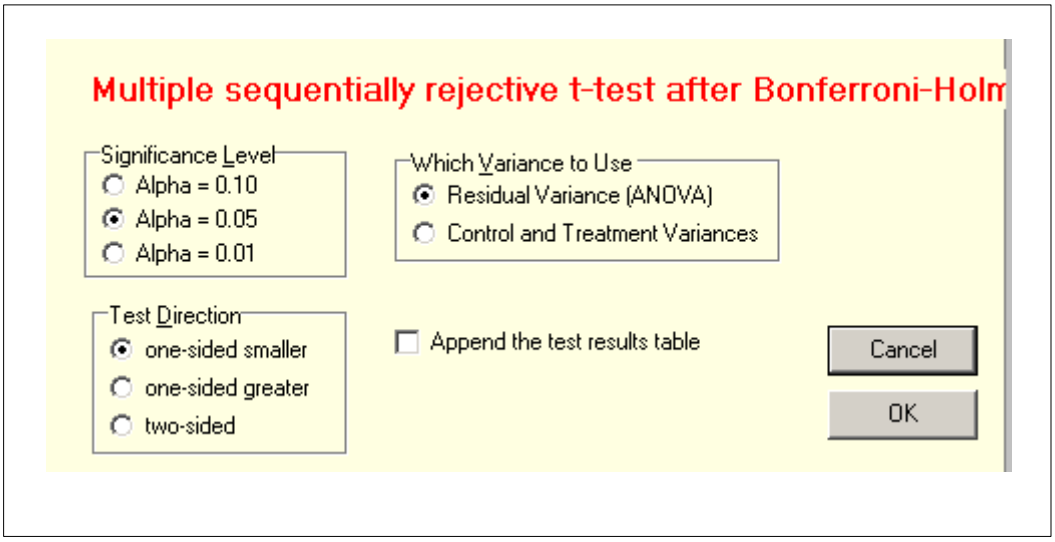


Figure 40: Dialog for specific statistical procedures under “Statistical Procedures”

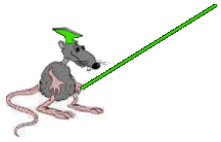
Presets are: “Significance Level”, (= “Alpha”, “Error Probability”), “Test Direction” and — in some parametric tests — “Which Variance to Use”. You don't have to make any changes here; if no settings are changed, then the software recommendations are applied. The "Significance Level" is set to 1% (0.01) for pretests for normal distribution and variance homogeneity. All other tests are conducted with a significance level of 5% (0.05) as standard.

Some specific tests are two-sided as standard due to their type of questioning. Where that is the case, then the relevant check box will be deactivated, i.e. you will not be able to change that setting. In all other cases: For metric variables, the preset test direction is “one-sided smaller”, for quantal variables (e.g. mortality) the setting is generally “one-sided greater”. These settings can be modified as needed.

For two-sample and t tests, you can decide whether to use “Residual Variance” from an upstream ANOVA, or whether the individual “Control and Treatment Variances” should be used. The option with most statistical power is preset. In most parametric tests, the variance to be used is dictated by the test itself; if that is the case, then the selection box is greyed out.

All framework conditions of a test set in this dialog are named in the relevant legend of the result table. Clicking “OK” will start the analysis.

To make things easy for you, we have added the check box “Append the test results table”. The results of earlier analyses with the current data set will be deleted ahead of the next analysis if this box remains unchecked, and you will receive an output of the currently selected analysis only. With the check box activated, the next result tables will simply be appended to the previous results.



With the check box "Append the test results table“ activated, subsequent test results will be appended at the end of the previous test results for the same data set. This feature allows you to collect, save, and report the results of several statistical tests in one single file .
Previous results will be deleted with the box unchecked, and the file will contain only the results of the currently selected procedure.

4.7.1 Statistical Characteristics ("Simple Statistics")

An overview table with the following key indicators for each treatment is created via this menu item: Mean value, median, minimum, maximum, sample size, standard deviation, coefficient of variation, standard error, standard error percentage, 95% confidence interval, upper and lower confidence limits. Available only for metric data or replicated quantal data following transformation.

4.7.2 Analysis of Variance

ANOVA one-way Kruskal-Wallis Test Metric data, replicated quantal data following transformation	Exact r x 2 Table Chi² r x 2 Table Quantal Data

Figure 41: Available statistical methods for the variance analysis of a) metric or transformed quantal data, and b) quantal data.

A variance analysis allows you formulate a general statement of whether or not the various treatment groups of a data set differ significantly. The variance analysis does not allow an extrapolation of which treatment group may be significantly different from the control. The test will always be two-sided, which means that a variance analysis will always have less statistical power than a one-sided statistical test. It is therefore quite possible to find significances in a subsequent statistical test, even though the variance analysis returned no indication of an effect.
Select a single-factor variance analysis (“ANOVA one-way”) for metric (or transformed quantal) data with normal distribution and variance homogeneity. Alternatively, use the

Kruskal-Wallis test as non-parametric ANOVA for data without variance homogeneity and normal distribution.
The analog procedure for quantal data is the exact contingency table ("Exact r x 2 Table"). This procedure has its limits for very large numbers; ToxRat will output a relevant warning when the limit is reached. If that is the case, then you should apply the Chi2 contingency table ("Chi2 x 2 Table") – which is a normal distribution approximation of the contingency table for large numbers.

4.7.3 Analysis of Variance plus Trend

Jonckheere-Terpstra Test Metric data, replicated quantal data following transformation	Cochran-Armitage Test Quantal Data

Figure 42: Available statistical methods for the variance analysis plus trend analysis of a) metric or transformed data, and b) quantal data.

The Jonckheere-Terpstra and the Cochran-Armitage test — just like a variance analysis — allows you to determine whether or not the various treatments of a data set differ significantly. In contrast to the ANOVA or contingency table, the analysis may be, however, also single-sided, i.e. an increasing or decreasing trend is postulated ahead of the analysis and is then specifically tested to verify, whether or not the data significantly follows the postulated trend.

Where a trend is proven, the condition of monotony, which is prerequisite for many statistical procedures, is fulfilled.
ToxRat additionally offers a contrast analysis (as explicitly required in the OECD Test Guideline 210) to prove monotony.

4.7.4 Testing Normal Distribution

Shapiro-Wilk's Test
Kolmogorrof-Smirnov Test
Range-by-Standarddev. Test

Figure 43: Available statistical tests for normal distribution for metric data or quantal data following transformation.

Normal distribution is an important prerequisite for so-called parametric test procedures (see also Figure 21). Normal distribution tests are therefore available only for metric data or replicated quantal data following transformation. For the latter, you should rate any deviation from the normal distribution found under the premise that the objective of transformation is to create normal distribution.

The Kolmogoroff-Smirnow test is available only for a sample size equal to or larger than 4; the R/S test is provided for smaller sample sizes (sample size = number of replicates per sample). Both, however, have the drawback that they test each treatment separately, which might make it difficult for you to define a definitive overall statement.
The Shapiro-Wilks for normal distribution is a ToxRat default setting, because it analyses the entire data set as a whole, and therefore always delivers a definitive statement, and because it works independently of sample size (= number of replicates) and number of samples (= number of treatments).

4.7.5 Testing Variance Homogenity

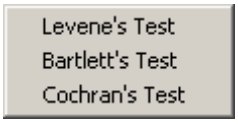
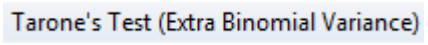
 Metric data, replicated quantal data following transformation	 Quantal Data

Figure 44: Available statistical tests for variance homogeneity for a) metric data or quantal data following transformation and for b) quantal data.

Variance homogeneity is an important prerequisite for so-called parametric statistical tests (see also Figure 21). Variance homogeneity tests are therefore available for metric data or replicated quantal data following transformation. Please note for the latter that the objective of transformation is to obtain variance homogeneity — which is why it will be redundant to test for variance homogeneity, and any variance heterogeneity still found should be viewed with a critical eye.
The Bartlett test is meant for normal distribution data with more than 10 treatments, while the Cochran test can be applied for less than 10 treatments, and does not require normal distribution. Both, however, have the drawback that they test each treatment separately, which might make it difficult for you to define a definitive overall statement. The Levene test for variance homogeneity is a ToxRat default setting, because it analyses the entire data set as a whole, and therefore always delivers a definitive statement, and because it works independently of the size or number of samples.
Replicated, quantal data are investigated for extrabinomial variance by Tarone test. When indicated, a subsequent step down Cochran-Armitage test then is performend using the so called Rao-Scott variant, which takes into account the extrabinomial variance. Whenever you select the Cochran-Armitage-test, ToxRat automatically checks for extrabinomial variance.

Since variance homogeneity and normal distribution are basic prerequisites for the use of powerful main tests, we recommend using an error probability of 1% to decrease the risk of false positives in pre-tests (and therefore unnecessarily weak main tests). More importantly, you will avoid so-called “alpha cumulation” , i.e. the cumulation of error probabilities within the complete sequence of pre-tests and main test.

4.7.6 Analysis of Monotone Trend by Contrasts

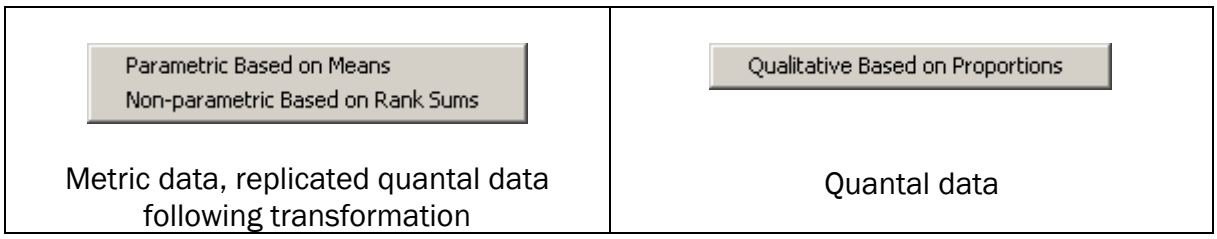


Figure 45: Available statistical methods for monotone analysis of a) metric or transformed quantal data, and b) quantal data.

Some statistical methods for monotone analysis require that the variable for analysis — despite any eventual random scattering upwards or downwards — as a rule display a monotonous dose-response-relationship.
One statistical procedure to establish that fact is contrast analysis. Contrast analysis is a special variation of the variance analysis, which not only tests whether or not significant differences exist between treatments, but also whether these differences display a specific trend. As variance analysis, the contrast analysis is performed as a twosided test as a rule. To say it as simply as possible: the original data is calculated with fixed coefficients, which are selected in order to specify a postulated trend. A subsequent test will then verify, whether the resulting values (contrasts) match the postulated pattern. ToxRat will carry out the contrast analysis for linear, as well as square contrasts, as specified in e.g. Test Guideline OECD 210. **The monotony requirements are regarded as satisfied if at least the linear trend is significant.** The contrast analysis is based on mean values for metric data with normal distribution and variance homogeneity; should these prerequisites not be fulfilled, then the rank sums will be used. For quantal data, the relevant percentages are used.

4.7.7 Two-Sample Comparisons

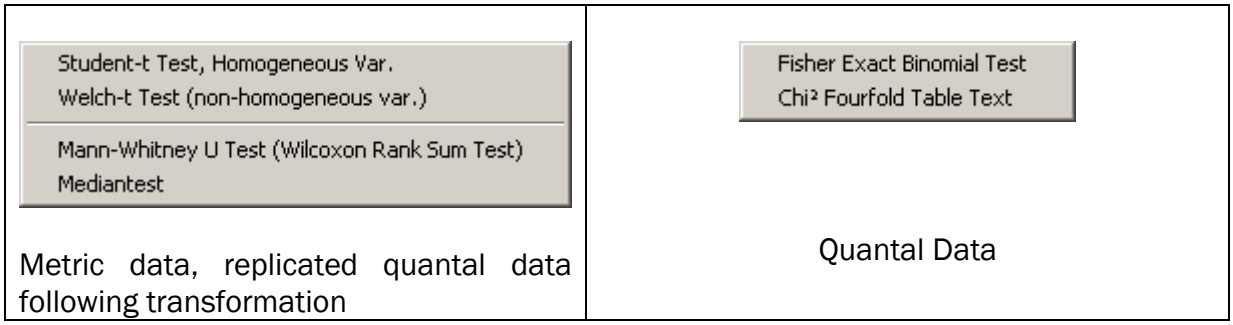
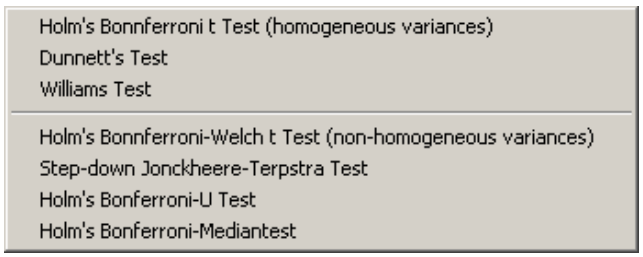


Figure 46: Available two-sample tests for a) metric or transformed data, and b) quantal data.

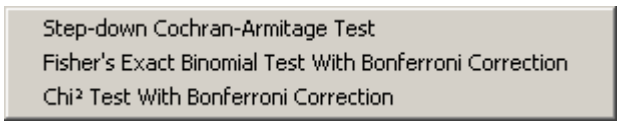
Here you will be able to carry out a specifically selected two-sample test. A dialog will open once you have selected a procedure, and you can specify test direction and error probability (see also Figure 40). It would be a good idea to go ahead and read the section on NOEC testing (chapter 4.4) on the prerequisites and relevant properties of the various tests

("Which Test to Use When") now – the backgrounds and operating steps described apply for the two-sample testing procedure described above. Please note that the software will not automatically test for prerequisites in this case.

4.7.8 Multiple Comparison with a Control



Metric data, replicated quantal data following transformation



Quantal Data

Figure 47: Available multiple tests for a) metric or transformed data, and b) quantal data.

Where multiple treatments are to be compared with the control to e.g. extrapolate an effect threshold, we can't simply carry out several two-sample tests one after the other, because the individual error probabilities would accumulate (alpha cumulation). Instead, we will either use specific multiple tests (Dunnet test, Williams test, Step-down-Jonckheere-Terpstra test, Step-down-Cochran-Armitage test), or we can modify specific two-sample tests using the so-called Bonferroni Correction. Multiple tests will ensure that the overall error probability for all comparisons will not surpass the selected error probability. The Bonferroni Correction in particular, will decrease the statistical power. Please read chapter 4.4 to find out more about statistical power and which test to apply when.
A dialog will open once you have selected a test, and you can specify directionality and error probability for the multiple test (see also Figure 40). Please note that the software will not automatically test for prerequisites in this case.

4.7.9 Outlier Tests

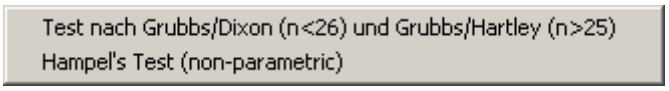


Figure 48: Available outlier tests for metric data

It can happen on occasion, that a measurand does not match the rest of the sample - when that happens it may be useful to explore, whether that value nevertheless could be regarded as part of the total population of data. If the answer is yes, then the deviation is within the scope of natural variability. If the answer is no, then we are looking at a so-called outlier.
An outlier test verifies the probability of an extreme value being part of the total population of data.

ToxRat will carry out outlier tests, but any values suspected to be outliers will not be automatically eliminated by the software. Any specific measurand out of the data set not to be included for additional analyses must be deleted manually in the raw data input form.

ToxRat includes the Grubbs test for normally distributed data, and the Hampel test for non-normally distributed data. Based on the mean values (Grubbs) or medians (Hampel), a parameter is calculated and tested for the relevantly largest and smallest value of each treatment to verify whether it is within the limits of the total population of data. The relevant limit values are listed in tables depending on the sample size.
ToxRat will carry out the Grubbs test automatically in the variation according to Dixon or Hartley depending on sample size. The Hampel test cannot be carried out if more than 50% of the sample data display the same numerical value.

4.8 Handling a Solvent Control

If your biotest includes a solvent control, enter the test result as a rule next to the control in the second column of the data entry sheets. Add the name "Solvent Control" or simply "Solvent" (see Figure 49).

Raw Data (given as: cells/ml)

Time	Treatment	µg/L				
96,0 h	Control	Solvent	0,038	0,068	0,12	0,21
1	280,0	287,0	49,5	16,0	7,0	1,5
2	234,0	256,0	33,5	16,0	5,5	3,0
3	218,0	154,0	24,5	13,0	6,0	3,0
4	208,0	204,0				
5	158,0	158,0				
6	238,0	214,0				
#Replicates	6	6	3	3	3	3
Mean	222,67	212,17	35,83	15,00	6,17	2,50
Std.Dev	40,17	52,74	12,66	1,73	0,76	0,87
CV%	18,0	24,9	35,3	11,5	12,4	34,6

Figure 49: Data input where solvent control exists

ToxRat will then recognise the existence of a solvent control and will automatically offer a selection dialogue before each statistical evaluation, where you can specify how to handle the two controls (Figure 50).

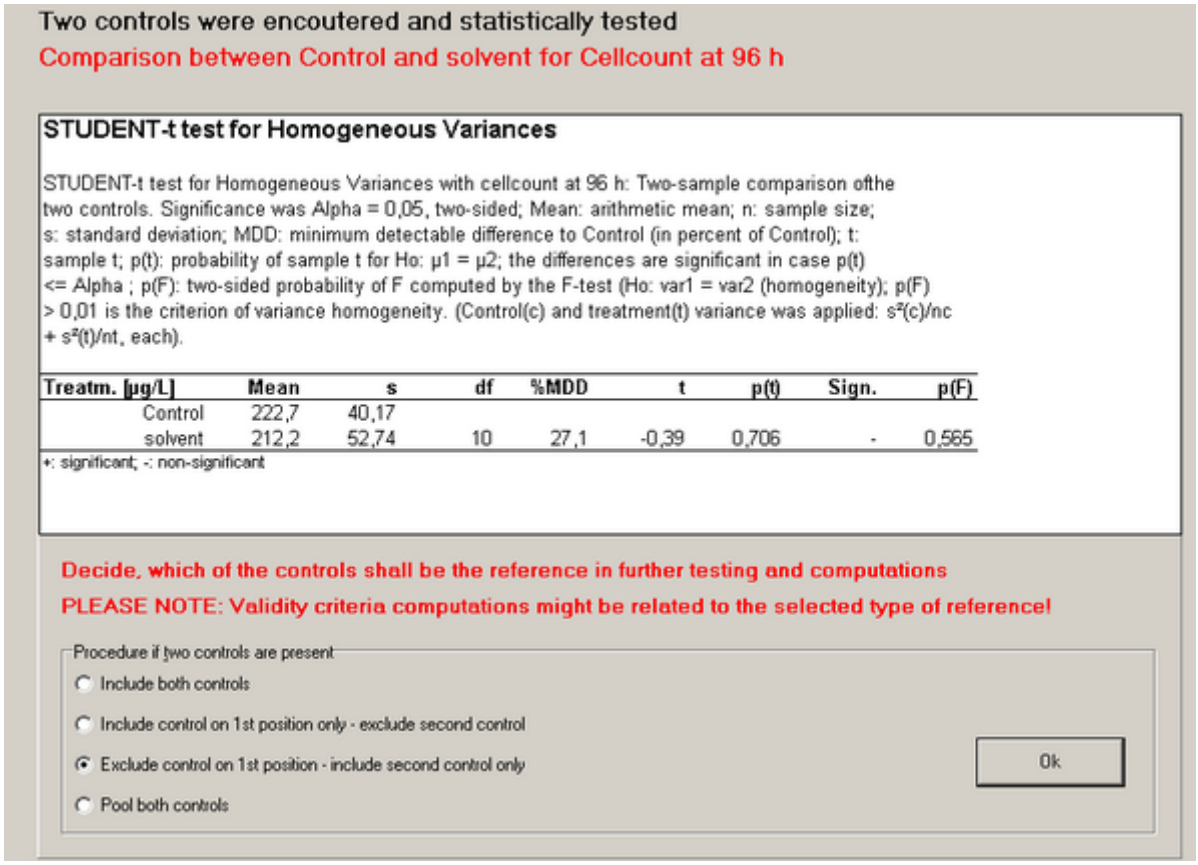


Figure 50: Selection dialogue for the handling of a solvent control

ToxRat will first present the result of a two-sample test between control and solvent control. Metric data will trigger a two-sample Student-t test, for quantal data a Fisher test or Chi2 test will be carried out. You can then select from the following options to go forward:

• Include both controls	The benchmark for finding effect levels and statistical tests is the first control; the second (solvent) control is included in statistical evaluations as a standalone treatment
• Include control on 1st position only - exclude second control	The benchmark for finding effect levels and statistical tests is the first control; the second (solvent) control is not included in statistical evaluations
• Exclude control on 1st position – include second control only	The benchmark for finding effect levels and statistical tests is the second (solvent) control; the first control is not included in statistical evaluations
• Pool both controls	First and second (solvent) control are pooled and form a common benchmark for finding the effect level and for statistical evaluations

In Biotest Workbooks with several variables, a selection dialog related to solvent control is displayed for each variable. That means you can specify for each individual variable, how the solvent control should be handled.
The data basis for two-sample tests will always be the last measuring interval. Your decision of which control to use will then be applied for the statistical evaluation of all measuring intervals.

The validity check generally is based on the first control – regardless of which control is selected as benchmark for statistical evaluations.

4.9 Validity Check

When a Biotest Workbook is used, then ToxRat will automatically carry out a validity check based on the validity criteria specified in the test guideline. In the options menu, you will find the validity criteria in a separate, biotest-specific dialogue with the relevant name of the guideline (see also chapter 5).

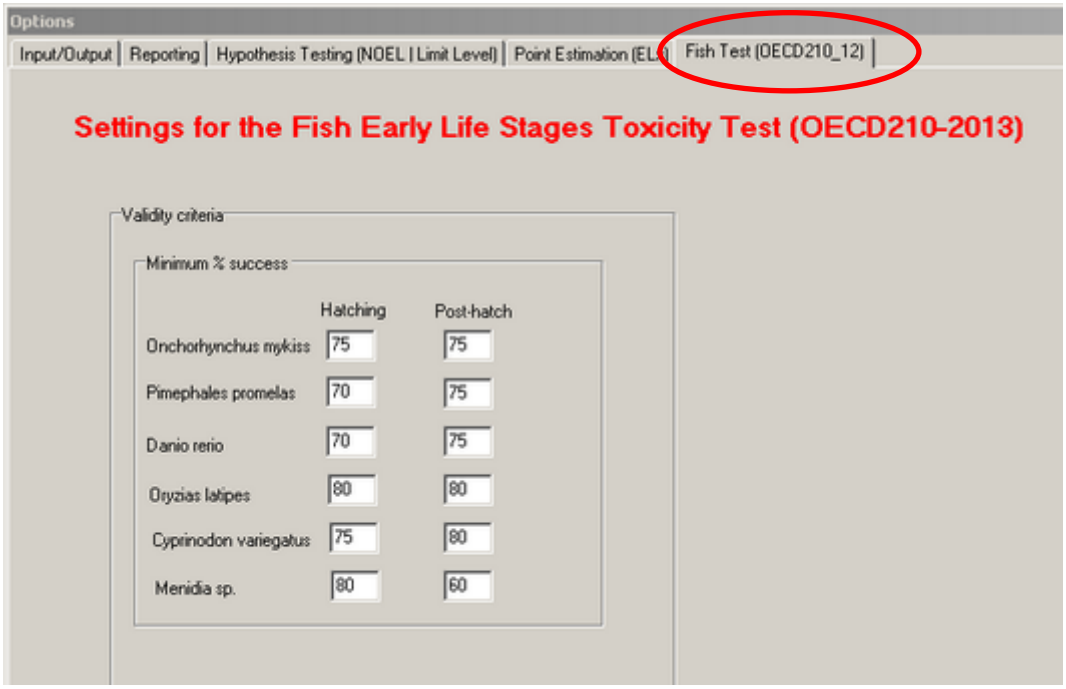
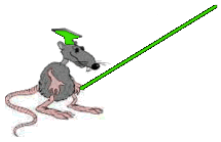


Figure 51: Biotest-specific dialogue for setting validity criteria, Example: OECD 210

The validity criteria specified in the test guideline are default values, and can generally be applied without changes. You are free to edit the validity criteria in cases where the test guidelines leave some options unspecified (e.g. where test organisms are used that are not listed in the test guideline, let's say OECD 201), or you want to explicitly use other than the default values. ToxRat will output the result of the validity check in a comparison of the criteria values and actual values found (Figure 52).

The test with Danio rerio requires a minimum hatching success of 70% and a minimum post-hatching success of 75% in the control to be valid. In the present test 95,0% and 94,7%, respectively, of the introduced fish did survive; thus the test is valid.

Figure 52: Result of the validity check as part of the result output, Example OECD 210



For selected biotests (e.g. OECD 201, OECD 221, ISO 20079), ToxRat will both list the validity criteria specified in the guideline ("required") and those currently set by the user ("user") in the validity check, to ensure that any changes performed by the user will be immediately visible. This function is, however, not (yet) available for all biotests.

In case there are several test guidelines available for certain biotest (e.g. algae growth inhibition test), the validity criteria of all guidelines are available in the biotest dialogue under "Options". Only those which are relevant for the currently used workbook will be available for editing. These are highlighted in a white background field.

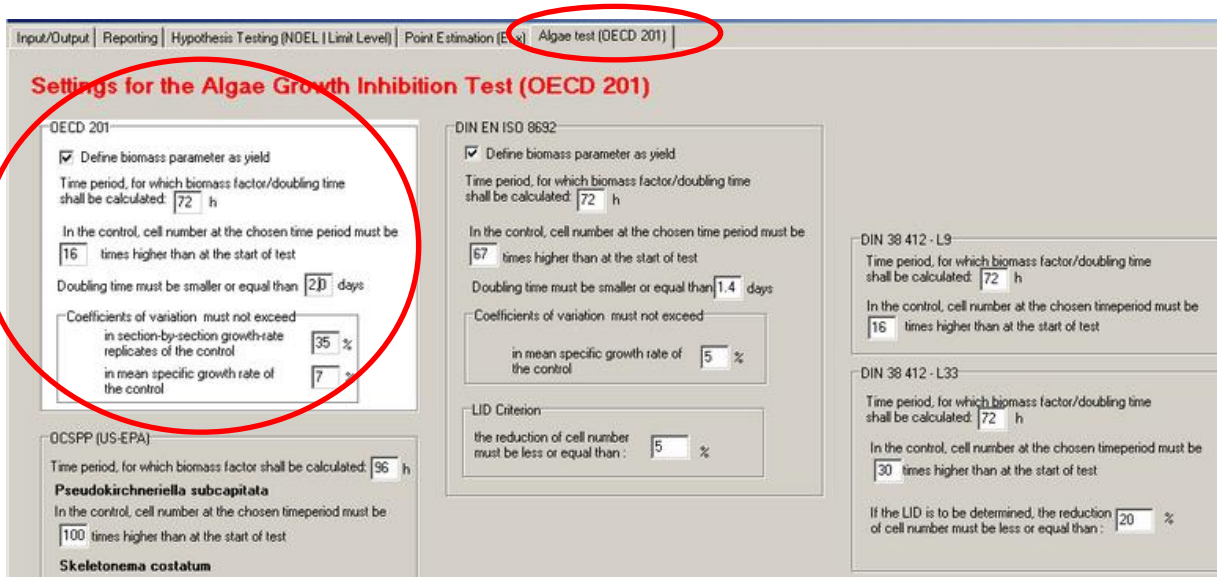


Figure 53: Setting dialogues for validity criteria in biotests for which several guidelines exist. White background: Guideline of the currently selected Biotest Workbook. Example: Algae Growth Inhibition Test.

The validity check generally is based on the first control – even if a solvent control exists and regardless of which control is selected as benchmark for statistical evaluations.

5 Biotest-Specific Settings and Information

Every Biotest Workbook enables testspecific settings using the menu “Options / <biotest name>”. Usually here only the validity criteria are shown (see chapter 4.9). However, some Biotest Workbooks allow special settings for evaluations, they contain specifics that must be taken into consideration or there are different workbook variants available. These are discussed in the following chapter.

5.1 Algae Growth Inhibition Test (OECD 201, OCSPP 850.5400, DIN 38412, ISO 8692)

The often very high values of algae cells can be divided by a specific factor set in out in "Generic Notes" in cell B11 to make your data input and the ToxRat visualisation easier. The default value here is 10000 (Figure 54).
The "Generic Notes" in all Algae Test Workbooks also contains the cell B12, which is the number of measurement repetitions ("repeated measurements per replicate"). If the value entered here is greater than 1, then ToxRat will interpret the relevant number of columns in the raw data sheet as measurement repetitions of the same concentration (Figure 54), for which a mean value will be determined. You will therefore have to make absolutely sure that the correct number of “repeated measurements per replicate” is entered in "Generic Notes". The value must be "1" if there will be no repetitions.

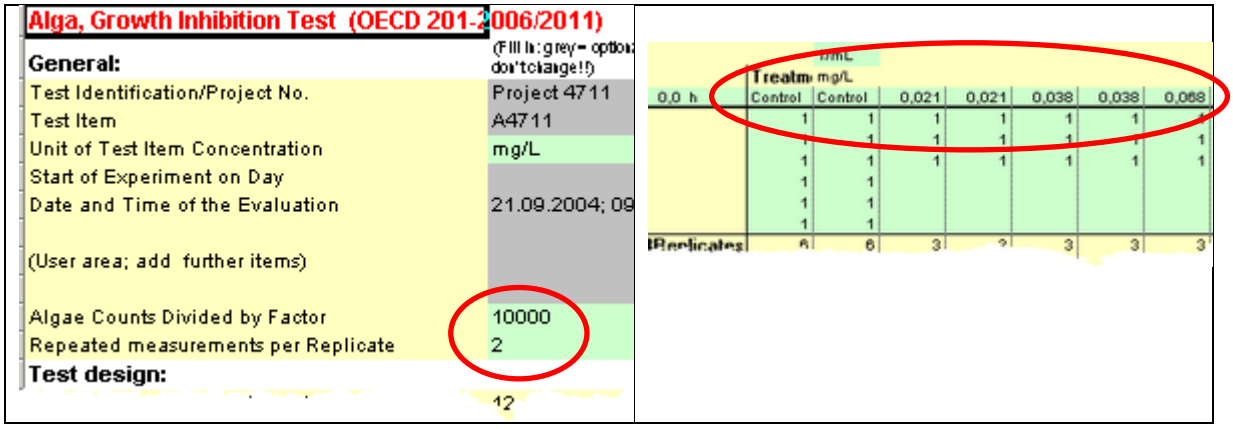
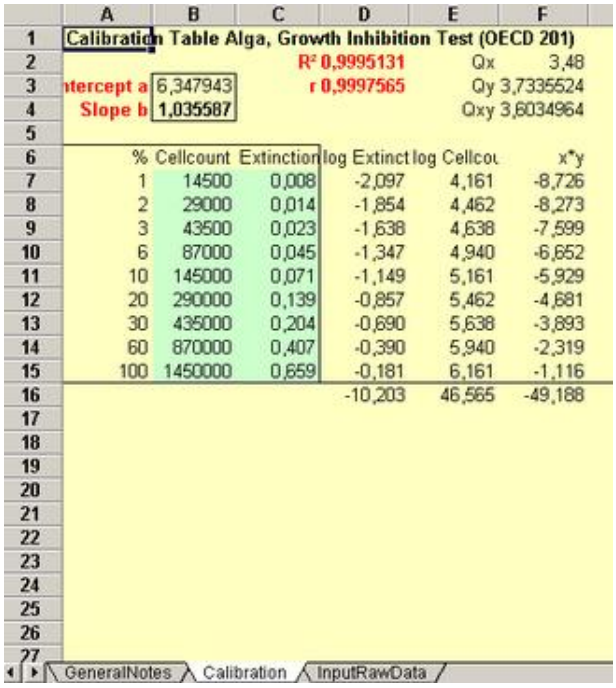


Figure 54: Data input for Algae Growth Inhibition Test if repeated measurements exist (in this case: number of repetitions = 2)

Check the number of repeated measurements entered in cell B12 of the "Generic Notes" if you notice that ToxRat is "skipping" evaluation treatments during an algae growth inhibition test. If the value entered there is too large and does not match the required structure for the data input sheet "InputRawData", then ToxRat will skip specific columns during the data read, as their values are not interpreted as individual concentrations.

Different workbook variants

Depending on whether you have measured the algal growth using cellcounts, extinction or fluorescence, you simply select a matching data input template (you will recognise these by their extension "cellcounts", "extinction", or "fluorescence").
In cell B17 of "Generic Notes" you can see whether the data input can be a direct cell count, or whether extinctions or fluorescences were measured - in which case the cell counts must first be converted by ToxRat. Important: You cannot make any changes here (cell encoding in yellow!). That means, if the workbook is designed for cellcounts, you must not fill in extinctions and vice versa. The fluorescence and extinction workbooks each contain an additional calibration data sheet (Figure 55). This data sheet is used for determining the equation needed for ToxRat to convert the measured extinction or fluorescence values into cell counts. Please enter your calibration values in the green cells. If you open the workbook in MS Excel, you will also be able to see a graphic representation of the calibration relationship in the relevant worksheet.



	A	B	C	D	E	F
1	Calibration Table Alga, Growth Inhibition Test (OECD 201)					
2				R^2 0,9995131	Qx	3,48
3	ntercept a	6,347943		r 0,9997565	Qy	3,7335524
4	Slope b	1,035587			Qxy	3,6034964
5						
6	%	Cellcount	Extinction	log Extinct	log Cellcou	x*y
7	1	14500	0,008	-2,097	4,161	-8,726
8	2	29000	0,014	-1,854	4,462	-8,273
9	3	43500	0,023	-1,638	4,638	-7,599
10	6	87000	0,045	-1,347	4,940	-6,652
11	10	145000	0,071	-1,149	5,161	-5,929
12	20	290000	0,139	-0,857	5,462	-4,681
13	30	435000	0,204	-0,690	5,638	-3,893
14	60	870000	0,407	-0,390	5,940	-2,319
15	100	1450000	0,659	-0,181	6,161	-1,116
16				-10,203	46,565	-49,188
17						
18						
19						
20						
21						
22						
23						
24						
25						
26						
27						

GeneralNotes | Calibration | InputRawData

Figure 55: Calibration data sheet in algae growth inhibition test workbooks for extinctions or fluorescences.

Additionally, ToxRat provides the following workbooks for special test- or measurement designs:
„OECD201 AlgaeGrowthInhibition(Cells 0 h – Extinction).xls“
Enables input of cell numbers for test start (day 0) and of extinctions for all other measurement intervals.
„OECD201 AlgaeGrowthInhibition(Extinction duo).xls“
Enables the usage of two different calibration curves for different ranges of extinctions

In the biotest-specific tab (Menu Options – Algae Test), you will find a field containing special options in addition to the validity criteria (see section 4.9) under "Options" >> "Algae

Test". You will usually not need to change any of the default values, but you will be able to see in Figure 56 which additional options are available.

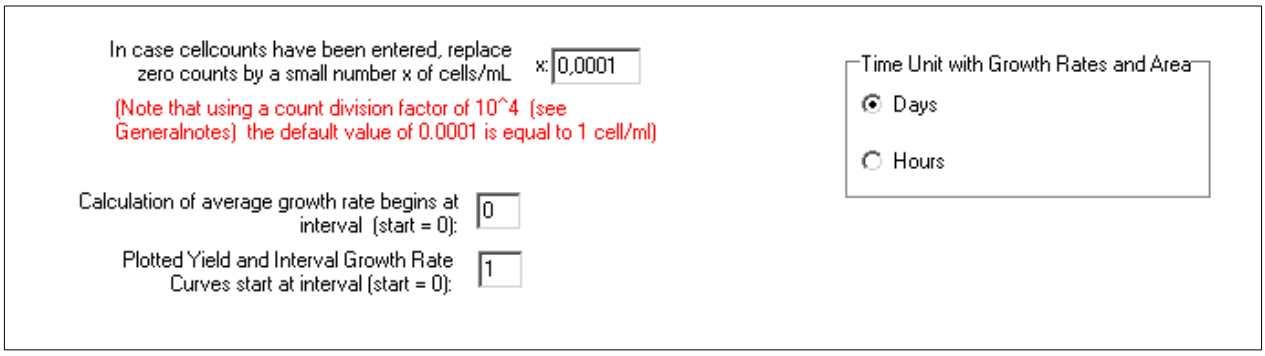


Figure 56: Special setting options for the Algae Growth Inhibition Test ("Options" >> "Algae Test")

5.2 Daphnia Reproduction Test (OECD 211)

According to the available test designs (semi-static and flow), and the optional measured variables specified in the test guideline, a number of workbooks for Daphnia Reproduction Tests are provided for you. Please select the template that matches your test type. All OECD 211 workbooks provide setting options under the biotest-specific tag of the option menu ("Options" >> "Daphnia Test"). You will generally not have to change any of the default values, but you will be able to see in Figure 57 which additional options are available.

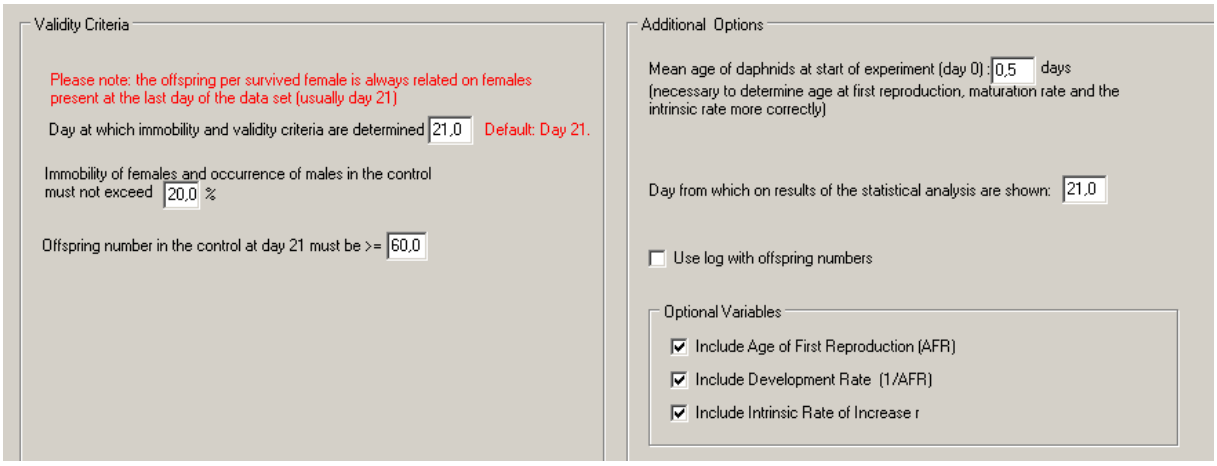


Figure 57: Special setting options for the Daphnia Reproduction Test ("Options" >> "Daphnia Test")

5.3 Chironomid Toxicity Test (OECD 218/219)

In a Chironomid test, a specific number of larvae is used for each treatment. The number of hatched males and females is counted for the duration of the test.
When a workbook template for the Chironomid Toxicity Test is opened, ToxRat will ask you what you want to do: either evaluating the existing data or entering new data (Figure 58). The reason behind is a special feature of the Chironomus data structure, which will be explained in this chapter.
Select “Data Input” to see the data structure of a Chironomus-workbook: it contains three data input sheets in addition to "Generic Notes":
„InputRaDataAll“,
„InputRawDataMales“
„InputRawDataFemales“.

The number of **larvae introduced** must be entered into the data input sheet "InputRawDataAll" for the measuring time Zero (green cells). The number of **males and females hatched** on each observation day must be entered into the data input sheets "InputRawDataMales" and "InputRawDataFemales". ToxRat will automatically fill in the relevant initial numbers and the totals of individuals hatched per day.

Before the data is evaluated, ToxRat creates an additional data sheet for each data input sheet containing the *cumulative hatched numbers* (identified by the file name suffix "-cum"), which are calculated from the daily hatched numbers in the data input sheets "InputRawDataMales" and "InputRawDataFemales". These data sheets also contain the individual numbers of introduced male and female larvae. Since the sex of introduced larvae is unknown, it is assumed that 50% of the larvae were male/female. All later data sheet evaluations will be based on the numbers in the data sheets containing the cumulative hatched totals!
An additional data sheet "InputSexRatio" is created for the evaluation of the male-female ratio.
To start an evaluation, press the refesh button and when youa re asked again what you want to do (Figure 58), then select “Evaluation”.

Automatic Data Consolidation

Based on the cumulative emerged numbers, counter errors when larvae were introduced become apparent, as the sum of all emerged cannot be greater than the number of larvae introduced. The following rule therefore applies: Once a Chironomid Workbook is evaluated in ToxRat, the software will check the data contained therein for consistency. You will be notified of the process by way of a relevant message prompt (Figure 59). Once you have confirmed to permit the process, a relevant status message will inform you of the result of the consistency check (Figure 60). Any inconsistencies found will be automatically corrected by ToxRat. The correct number of introduced larvae will be entered, and the corrected values will be written into an additional data sheet with the file name suffix "-corr". The sheets with the suffix –cum will be created anyway, regardless whether corrections were needed or not. If any corrections were needed, a corresponding detailed report about will be in the sheets with the suffix “-cum” (Figure 61). The additional data sheets "-corr" and "-cum" are furthermore easily recognised by the entry "sheet inserted by program" in cell H1.

All later evaluations of the data set will be based on the data sheets containing the cumulative hatched numbers and the sex-specific numbers of larvae introduced (which may be corrected values)!

ToxRat automatically saves the consolidated file along with its additional data sheets with the file name suffix "_modified" (in the same directory as the original file). You can therefore always be sure that there will be an original workbook available, which will only contain the cover sheet (Generic Notes) and the three data input sheets (see above). Of course you may also modify the name of the consolidated data file. ToxRat will not carry out any new data consolidation if a file with the suffix "_modified" is opened (or any data file which contains "-corr" and "-cum" - data sheets, respectively) (Figure 62). Hence, any changes of the raw data will not be accepted, **i.e. a file containing additional data sheets must not be used for data input.**

Data Input

Select your favourite from the following data input options:

Data input in a Masterbook using ToxRat, i.e. into an empty data template (File >>"New Masterbook", then import as workbook using "refresh").
Data input in a Demo Workbook using MS-Excel ToxRat, or in one of your own previously used workbooks without the suffix "_modified" (i.e. without additional data sheets) (access via File >> Open); any previous data in the workbook will be overwritten.

ToxRat recognizes, if a Chironomid workbook is opened which contains additional data sheets such as "_corr" and "–cum". In this case, ToxRat will not perform any more data consolidations and any changes of the raw data are not accepted. Therefore, files with the suffix "–modified" and / or with additional data sheets are not suitable for inputting new data.

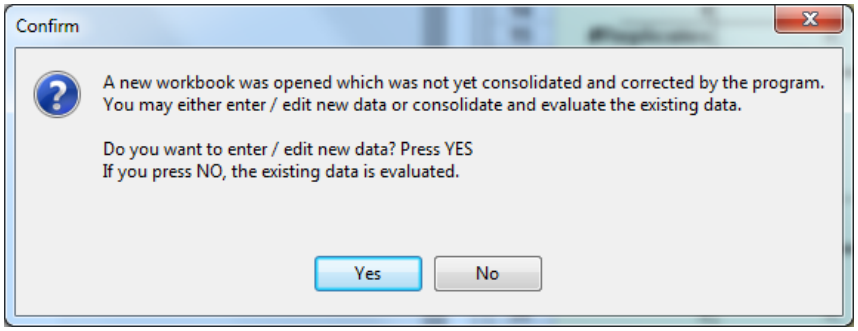


Figure 58: Automatic message displayed when a Chironomid Workbook is opened

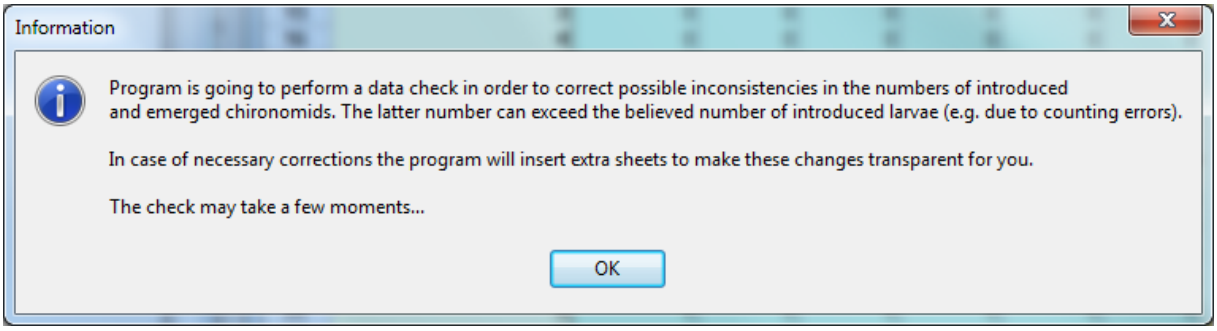


Figure 59: Automatic message before an evaluation is started.

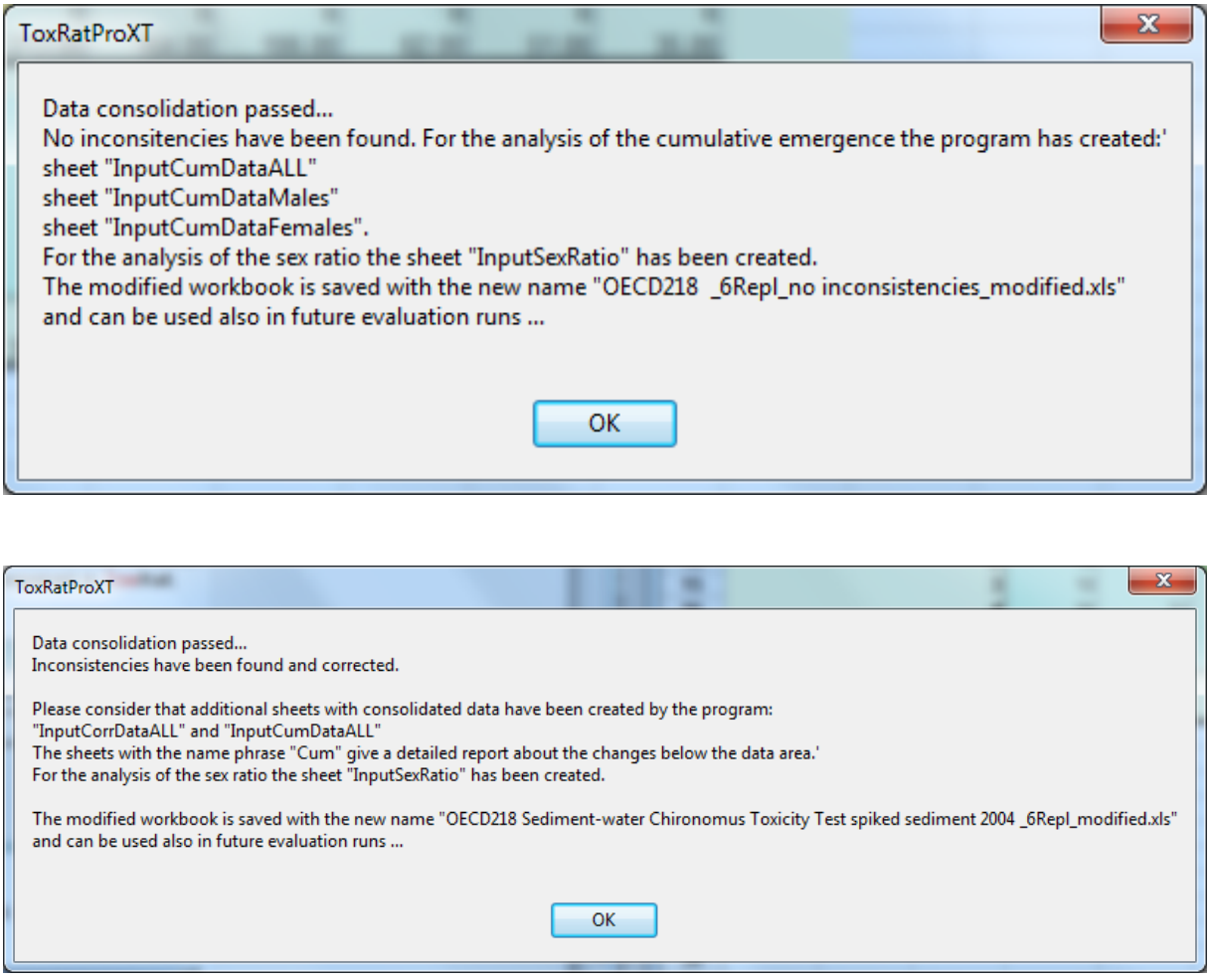


Figure 60: Status message about the result of ToxRat's automatic data consolidation for a chironomid data set. Sample data set without inconsistencies (upper box) and with inconsistencies (lower box).

140	4	19	18	12	7	4	4
141	#Replicates	4	4	4	4	4	4
142	#Emerged	70,00	71,00	54,00	36,00	28,00	20,00
143							

Report About Data Consolidation

Variables:
- Cumulative Emergence (All)
- Emergence (All, Corrected)

Consolidation Method:
In case more emerged than introduced chironomids are found,
the number of introduced is replaced by the number of emerged chironomids.
This is the only chance to calculate emergence rates and their toxic metrics.
The Development Rate/Time and Sex Ratio remain unaffected by this consolidation.
The changes made by the program do only affect the number of introduced larvae.
All remaining data remain unchanged.
Corrections are protocoled below and the change of data is indicated by bold numbers.
Sheet 4 (InputCumDataAll) Row 4 Col 5 Introduced Number 20 replaced by total emerged number 21
Sheet 3 (InputCorrDataAll) Row 4 Col 5 Introduced Number 20 replaced by total emerged number 21

Figure 61: Sample program report on a completed data consolidation in the Chironomid Workbook (can be found in the data sheets with the suffix "-cum" below the raw data).

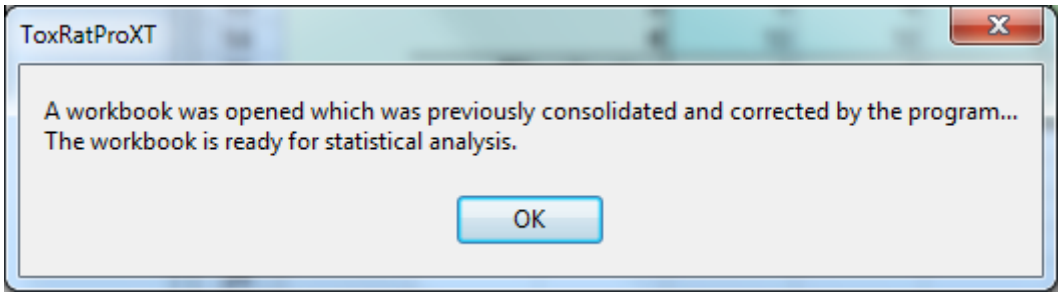


Figure 62: Message when opening a file with the suffix "_modified" or generally a file containing additional sheets resulting from data consolidation

There are some optional setting options available for the chironomid test, e.g. an evaluation of the male-female ratio, and a sex-specific evaluation of the emergence rates. An overview can be found in Figure 63.

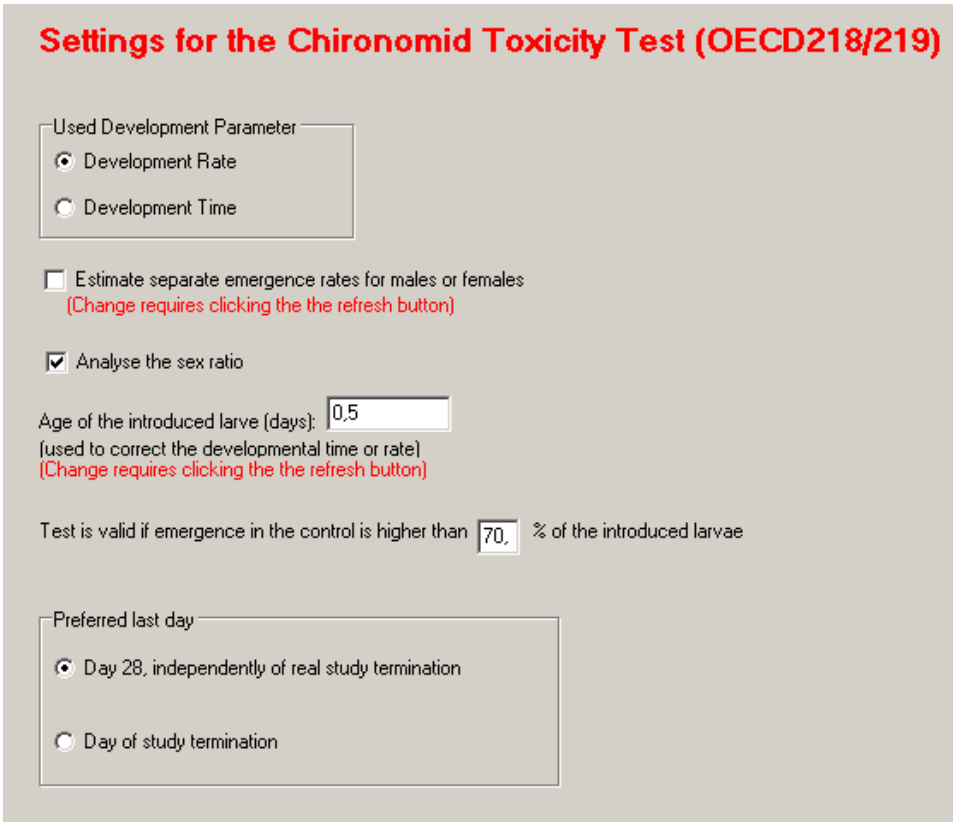


Figure 63: Specific setting options for the chironomid test (Menu >> Options >> Chironomid Test)

6 The Results

Or: What to find where and what does it all mean?

Our sample files are:
ToxRat Professional: Workbook OECD210
ToxRat Monitor: Workbook DIN EN ISO 15088
ToxRat Standard: „Testing a Metric response 3 at several Intervals.xls“.

ToxRat delivers the results in graphs and tables. This chapter will help you to find your way around ("What to find where....") and to understand and correctly interpret the results of the various statistical procedures (....and what does it all mean?").

Initiate the evaluation by clicking RUN (Biotest Workbook), or carry out a dose-response analysis (Generic Workbook). No matter which workbook you use or what statistical method(s) you chose - the general rule is: Start with having a look at the window displaying all the graphs available for each of the evaluation types. This will allow you to get a quick overall impression of the results, before you begin focusing on specific tables.
We'll get back to that later - go ahead and close the graph window for now (click "Close" or click the little "x" at the top right of the graph window), and let's have a look at the different types of information provided in the results screen.

6.1 Menus and Buttons in the Results Screen

Once ToxRat has completed an evaluation, new menu items and buttons appear in the menu bar for the evaluation (Figure 64).

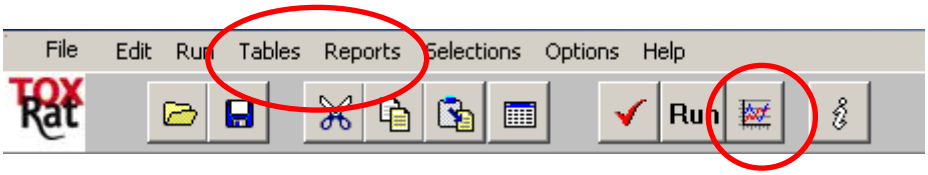


Figure 64: Menu item in the results screen

The following are newly added menu items for the results presentation:

Tables — List of all data and result tables - for the measured variables and for the derived variables, including "Yields" and "Growth Rates". Please see section 6.3 for more info.

Reports — Opens the report creation dialogue. This menu item is only available right here, since reports can only be created immediately after an evaluation. For more info on report creation see section 7.

 Reopens the graph window. For more info on the graphs see section 6.2.

6.2 Graphs

After each evaluation, ToxRat will initially display a window containing all graphs available for that particular evaluation (Figure 65). You can close that window at any time by clicking "Close" or the little "x" at the top right of the window - and reopen it by clicking the "Graph" button in the menu bar.

Graphs can only be generated immediately after an evaluation. They will be automatically deleted when the workbook is closed. If a workbook is saved, graphs will be never included. To save graphs, you will have to either generate a report (see chapter 7) or use the "Save" function in the graph editing mode.

There are two basic types of graphs: firstly a presentation of the measured variable against time, and secondly the result of the selected dose-response analysis via linear or non-linear regression. In the Biotest Workbooks with several variables, you can click the tabs to toggle between the variables.

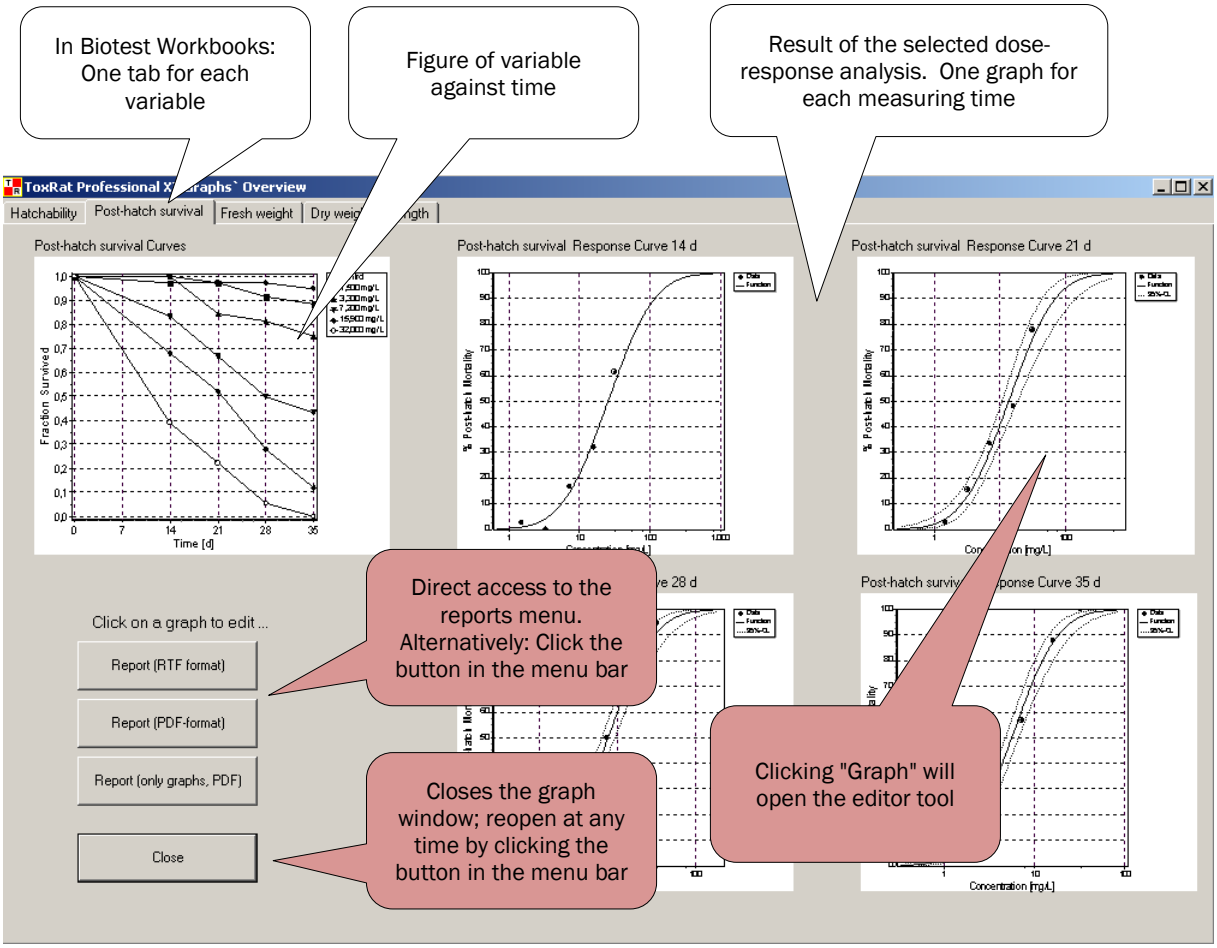


Figure 65: Structure of the Graph Window

Edit Graphs

Click the graph you wish to modify to enter the editing mode - the graph will be enlarged and offer its own menu bar (see Figure 66 and Figure 67). One of the many options is the menu item "Edit", which allows you to modify axis inscriptions or scaling. The editor is intuitive - just go ahead and try everything out. It is, however, only a relatively simple graph editor. If you need something more sophisticated - for publications, etc. - then you can export the graph data and use an external application (Figure 68).

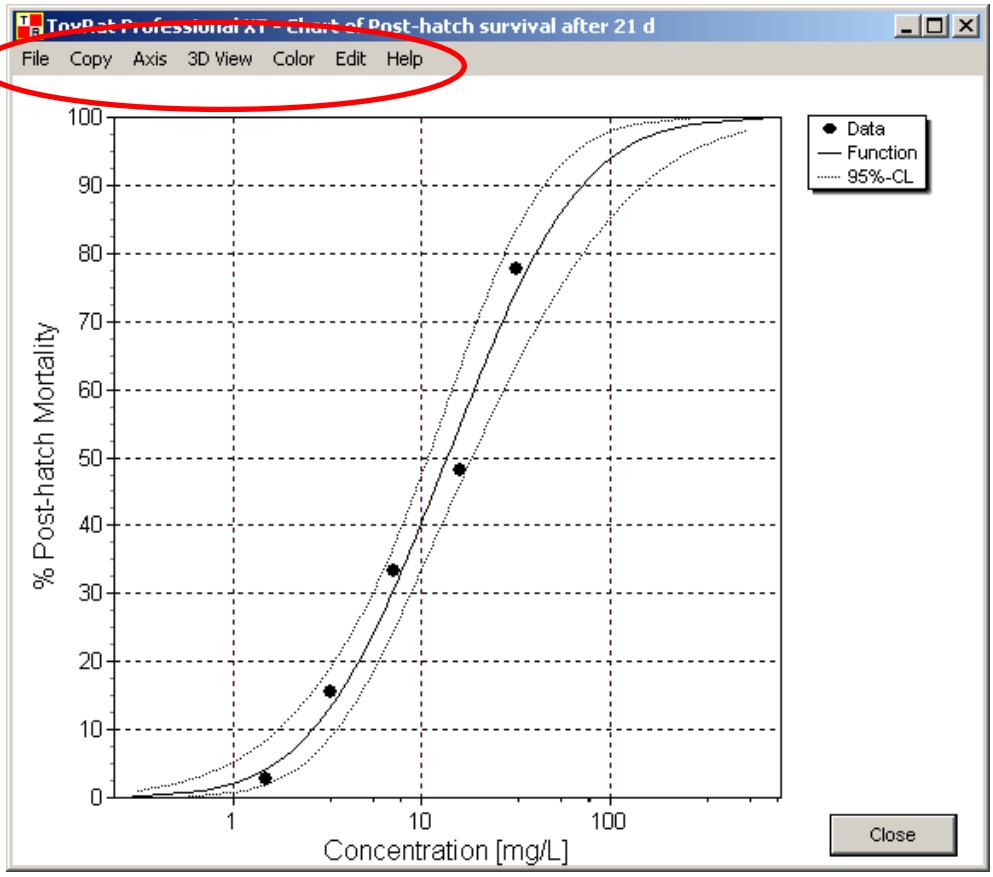


Figure 66: Edit mode for graphs – Overview

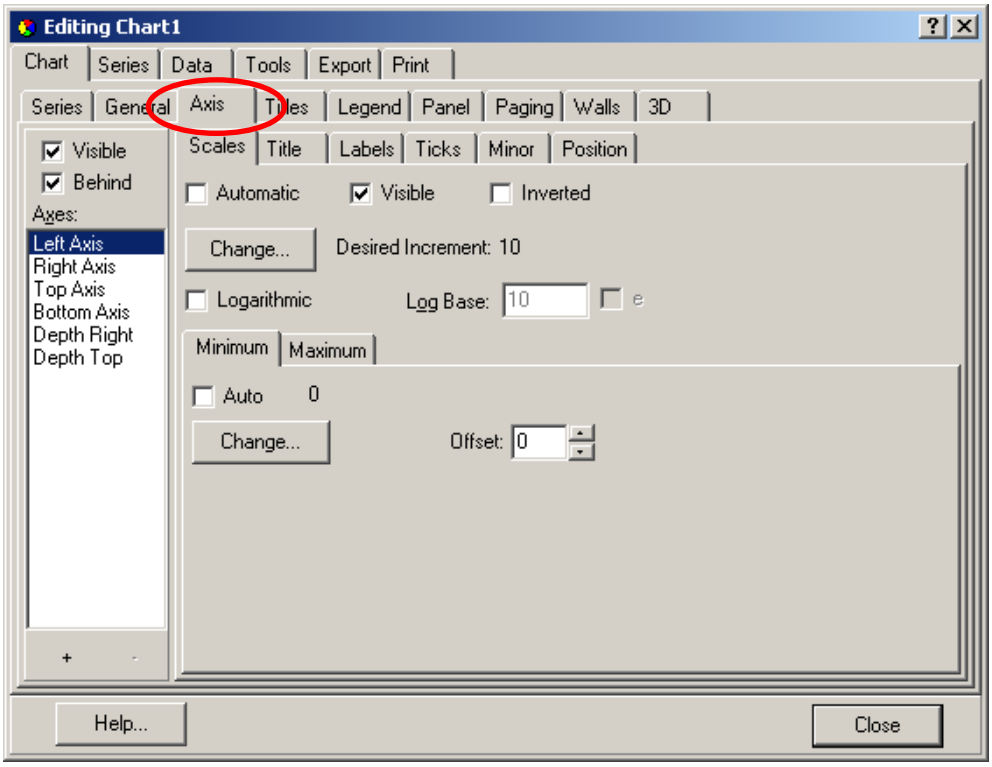


Figure 67: Edit mode for graphs - Axes (Edit >> Axis)

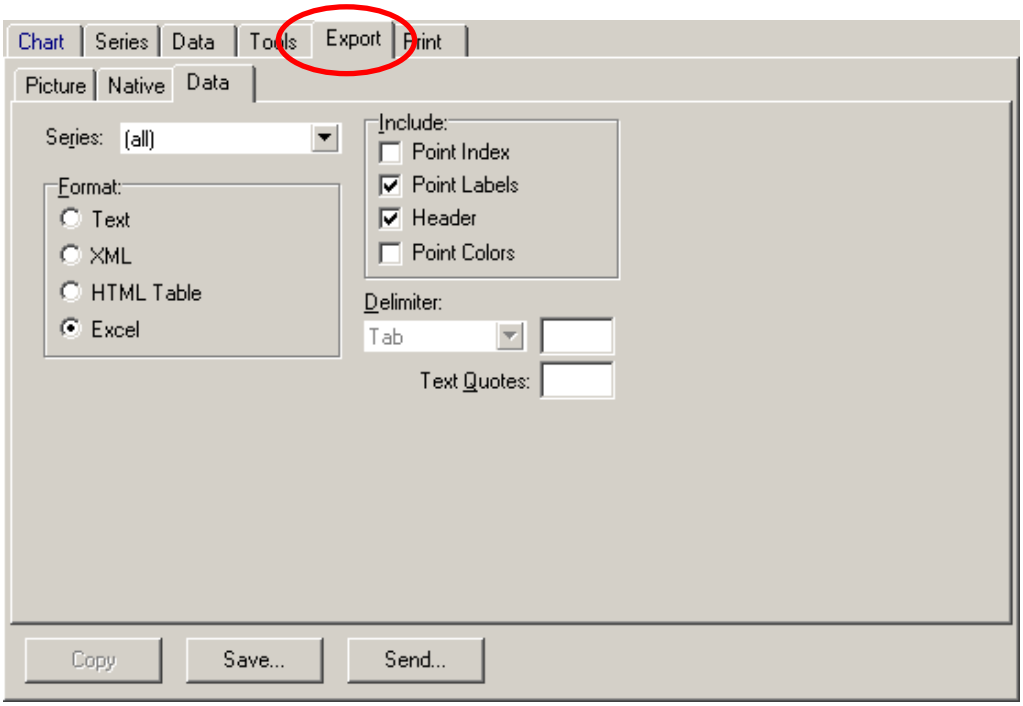


Figure 68: Edit mode for graphs - Export (Edit >> Export)

6.3 Tables

ToxRat will generate two types of tables for each evaluation: Data tables containing the (raw) data for evaluation, and result tables containing the results of a statistical procedure. Data tables are identified by the addition of "Data" to their name, and contain - depending on workbook - either the measured data from the data input sheets, or the derived data, e.g. cumulative numbers, yields, and growth rates, which ToxRat has calculated.

All evaluations are based exclusively on the data tables generated by ToxRat internally. This will ensure that ToxRat will always work with internally calculated sums, mean values, standard deviations, etc. - completely independently of the cell formulae used in the data input sheets for calculation or formatting.

Data and result tables are listed under the menu item "Tables" and can be selected and displayed individually. This list can be extremely long for complex biotests with multiple variables (Figure 69).

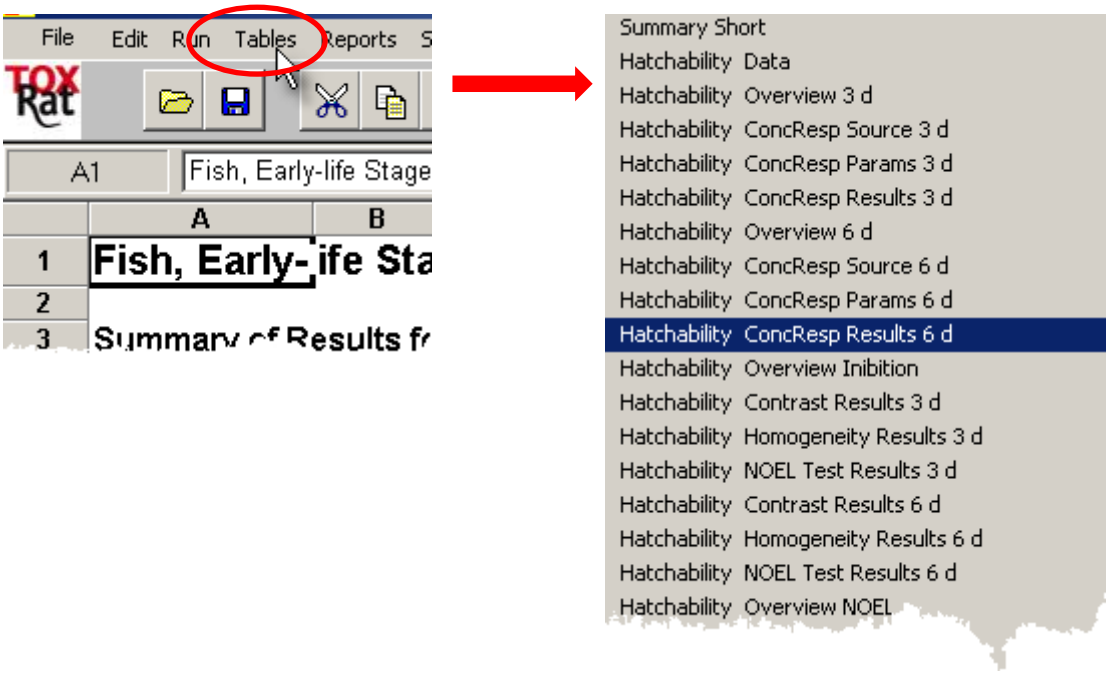


Figure 69: The menu item "Tables" contains a complete list of all tables generated for the current evaluation, and each table can be selected and opened individually

The good news is: You will generally not even need this list, because all result tables are additionally presented by ToxRat in additional sheets grouped by variable. These sheets are automatically inserted into the workbook (easy to find via the name “Tabs <Variable Name>”). (Exception: Table containing all EC values – see chapter Output of Non-significant and/or All EC Values 6.5.3).
For a Generic Workbook, in which only one variable is evaluated, there will only be one result sheet. For a Biotest Workbook several will be generated - one for each variable. There you will also find a sheet named "Summary", and one entitled "Settings" (

Figure 70) – we will discuss "Settings" in chapter 6.5.2. Clicking "refresh" will delete all result sheets.

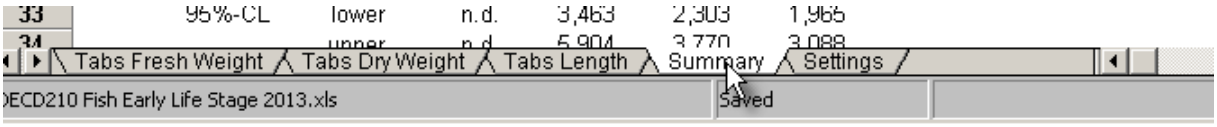


Figure 70: Newly added sheets after an evaluation (here: Workbook OECD 210)

A note for users of previous ToxRat versions:
From version 3.0 onwards, the data tables are no longer contained in additional sheets, but can instead be found under the menu item "Tables". In an effort to keep the file structure more manageable (particularly for very complex biotests), the additional sheets are now restricted to the result tables only.

You can save the file with all result sheets and reimport into ToxRat whenever you want to check the results. But don't forget: Graphs and reports will only be generated when a new evaluation is initiated!

Generic Workbooks display results in the sequence you chose for your evaluation(s). Biotest Workbooks display all results for a variable (i.e. in one sheet) in list form. The entries listed in a result sheet can be several hundred rows long depending on the number of measuring points to evaluate for one variable. You will have to scroll down to see all evaluation results. If you are looking for a specific evaluation result it is important to know that each result sheet follows the same basic structure (provided the relevant ecotoxicological end points are required by the corresponding guideline):

1. Dose-response analysis for measuring time 1 to x
2. NOEC determination for measuring times 1 to x. Thereby, for each measuring time, first the pretesting sequence is performed, followed by final testing.

ToxRat doesn't just deliver statistical endpoints or parameters - in the interest of better understanding, the results are also explained. For example: "p(F) is smaller than or equal to the selected significance level of 0.05; treatments are therefore significantly different". The individual steps in the sequence of pretesting and final testing for the NOEC requirement are also explained. You will find an example in Figure 71.

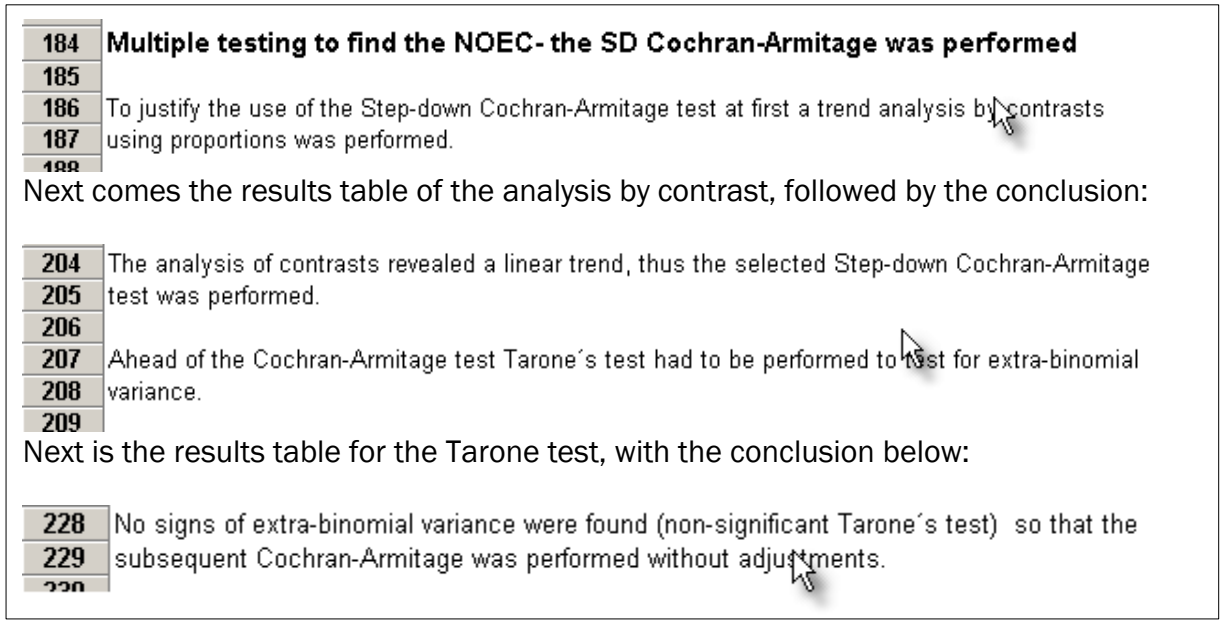


Figure 71: Sequence of explanatory texts and results tables (NOEC regulations for quantal variables, OECD 210, procedure in accordance with test scheme (see Figure 22, page 35)

ToxRat does its best to make the procedure for statistical analyses and the conclusions derived from the results as intuitive and easy to follow as possible. It is therefore advisable to take the time and read the table legends and texts provided for the results tables!

These and the table legends ensure that the results tables are for the most part self-explanatory. Additional hints are provided for selected tables below to help you understand how to use some of the information.

6.3.1 Table "Summary"

The table "Summary" contains the ecotoxicological endpoints (ECx, NOEC, LOEC, and poss. LID) for each evaluated variable and each measuring time. You can choose to have the relevantly used statistical method shown in the table; simply select it under Options >> Reports (Figure 72). This option is not automatically activated in the default settings.

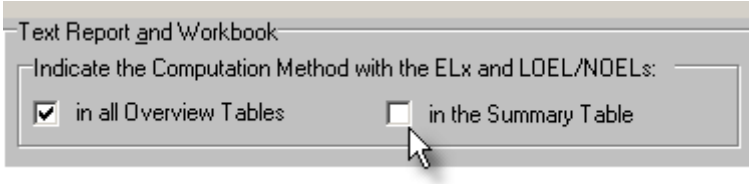


Figure 72: Selection dialogue for methodology display in results tables. Default setting: In overview tables "yes", in summary "no"

The results of the validity check can be found below the summary table alongside the conclusion of whether the test is considered valid or not.

6.3.2 Results Tables for Outlier Tests

Outlier tests are carried out separately for each treatment. The results table contains a sequential list of all treatments in rows, and there are two columns each for the significances of the relevant treatment: a check of the smallest and of the largest value. Sample reading (Figure 73): The smallest value of the control and the largest value of the 19.2 µg/L treatment are outliers with an error probability of 5%.

Outlier-test after Hampel

Outlier-test after Hampel with weight at 14,0 d: Significance level Alpha was 0,05: Med: median; MAD: median absolute deviation; 'n: sample size; Min, Max: minimum, maximum value of the sample; T_min, T_max: test statistic for Min, Max (after Hampel);' T*: critical margin for T_min|T_max; an outlier may be assumed, in case T_min|T_max >= T* (marked by a +sign).

Treatm. [µg/L]	Med	MAD	n	Min	T_min	T*	Sign.	Max	T_max	T*	Sign.
Control	50,00	2,545	18	35,58	5,6680	4,1	+	55,00	1,9630	4,1	-
1,200	49,00	3,005	10	42,00	2,3280	4,6	-	54,00	1,6660	4,6	-
2,400	41,00	2,000	9	35,89	2,5550	4,6	-	48,50	3,7500	4,6	-
4,800	35,00	3,000	10	31,00	1,3330	4,6	-	43,00	2,6670	4,6	-
9,600	27,50	2,500	10	23,00	1,8000	4,6	-	32,00	1,8000	4,6	-
19,200	19,00	1,000	8	17,00	2,0000	4,6	-	24,00	5,0000	4,6	+

+: significant (= outlier); -: non-significant
At least one, the minimum and/or maximum value was identified as outlier(+).

Figure 73: Example for a results table of the Hampel outlier test

ToxRat will as a rule not remove any data from the data set. Outliers can only be manually excluded from further evaluations by deleting them from the raw data table ahead of an evaluation (don't forget to click "refresh"!)

6.3.3 Results Tables for ECx Determination, Linear Regression

Four results tables are generated for each linear regression:

- Overview of the inhibition effects in percent
- Intermediate results from the Probit (or Logit, Weibull) analysis
- Parameters of the Probit (or Logit, Weibull) analysis
- Results: EC values and confidence limits

The parameter table contains important information on results assessment, the so-called "**criteria for goodness of fit**": P(Chi^2) (= unit of measure for the goodness of fit) and P(F) (= unit of measure for the significance of the dose-response relationship (Figure 74).

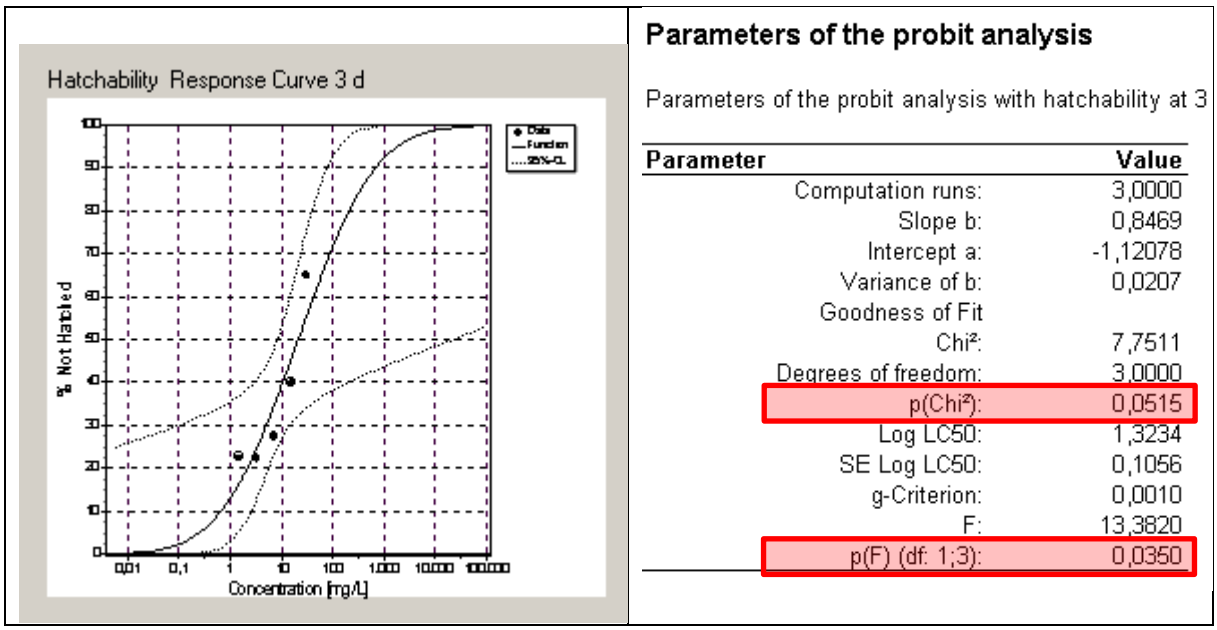


Figure 74: Example for a dose-response analysis for quantal data by way of linear regression; function selected: Probit; graph (left) – and associated parameter table (right) with important criteria for goodness of fit for the result evaluation

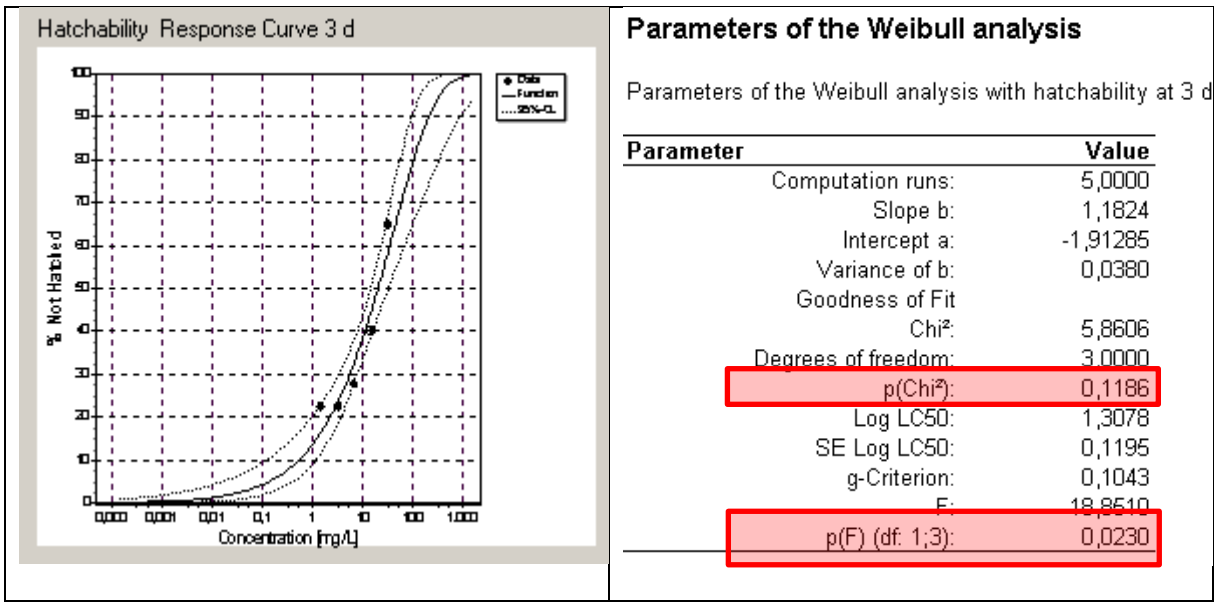


Figure 75: Example for a dose-response analysis for quantal data by way of linear regression; function selected: Weibull; graph (left) – and associated parameter table (right) with important criteria for goodness of fit for the result evaluation (using the same data as in Figure 74).

The Chi² test quantifies the agreement between fit and data. It can be expected that the data points are not one hundred percent on the curve - the question is, however, whether the observed deviations are simply by chance, or if the selected function was incorrect.

P(Chi²) refers to the probability of the data points being chance deviations. If p(Chi²) > 5%, then there is no significant deviation between fit and data.

The greater the value for p(Chi²), the better the fit (max. value is 1 (=100%)). If the value for p(Chi²) is smaller than 0.1, the so-called heterogeneity correction is triggered, which means that the confidence intervals are broadened to consider the greater data scattering (see chapter 4.6.1, for more details). In a favourable goodness of fit, p(Chi²) will usually be 0.7 or higher – if that is not achieved, then you should check if another function (Logit, Weibull) would possibly give a better fit (for our example, this was demonstrated in Figure 75).

Whatever the goodness of fit, ToxRat will check the significance of the resulting dose-response relationship in an F test. The F-Test checks the probability to obtain a dose response relationship from the sample data by chance - i.e. although in the basic population no dose response relationship exists. A significant dose-response relationship exists only if p(F) is smaller than 5%. Therefore, if p(F) is greater than 5%, i.e. the relationship found is non-significant, ToxRat will issue a warning (Figure 76) and usually no confidence intervals can be calculated. An output of ECx values may, however, be possible depending on the data situation. These can be helpful for orientation purposes, even if the EC values cannot not be reported due to their insignificance and non-existent confidence intervals. If you don't want that, you can easily change it in the "options" menu by deselecting "Input/Output" (Figure 77; the default setting is: Display ECx values and graphs).

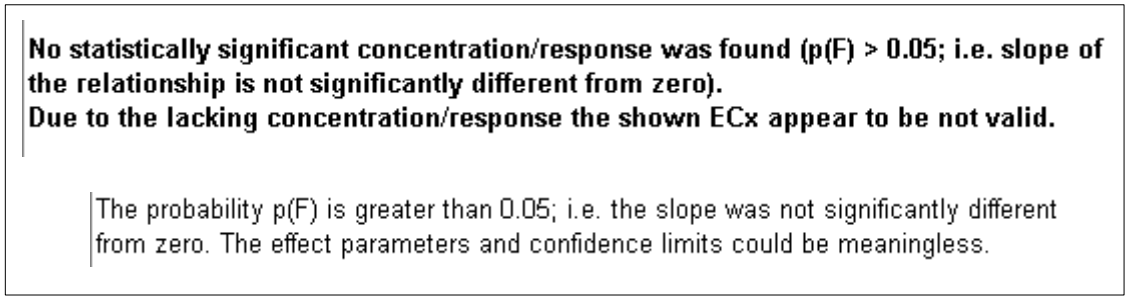


Figure 76: Warning regarding insignificance of fit

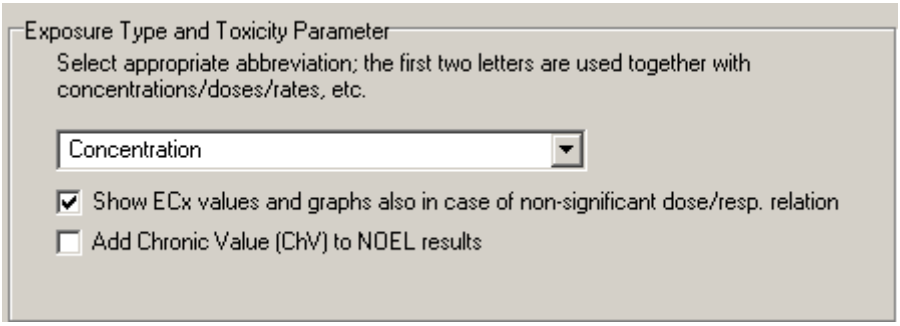


Figure 77: Setting option for the output of ECx values and graphs for non-significant dose-response relationships (menu Options >> Input/Output).

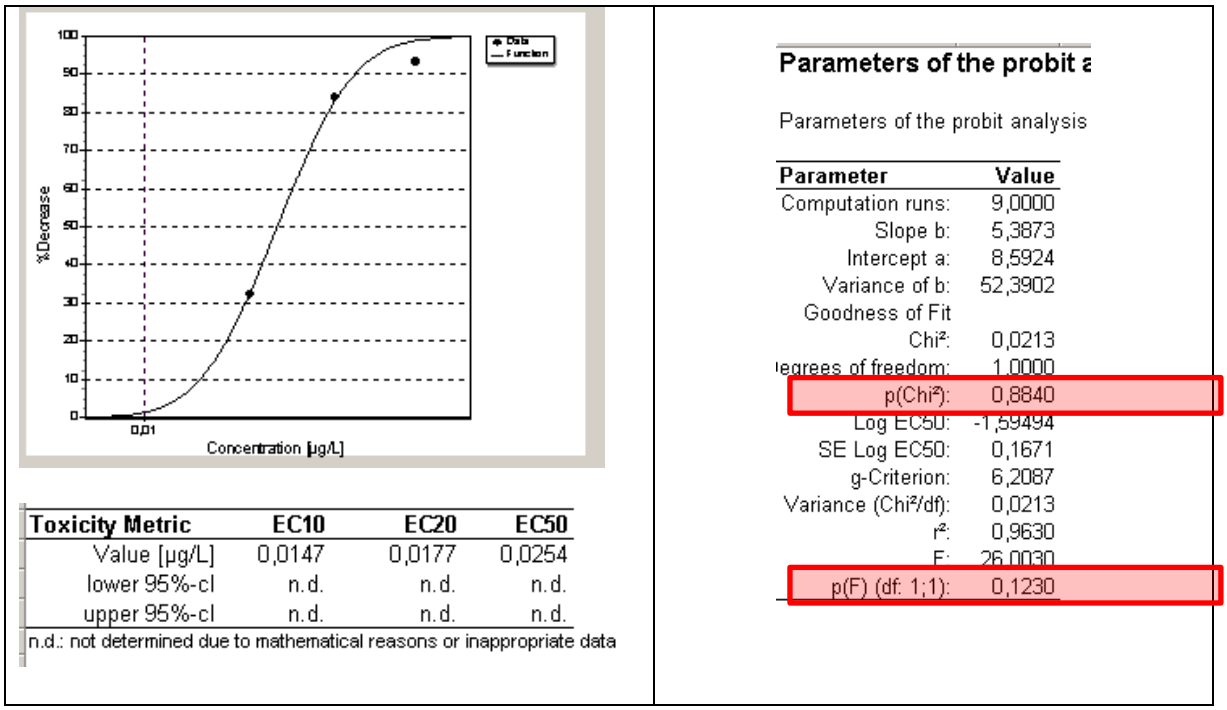
For some data situations it may seem surprising at first glance that no confidence intervals are shown, as apparently a clear dose-response relationship exists and the goodness of fit is high (Figure 78).
A look at p(F) will, however, reveal that the probability of three data points appear at their location purely by chance (i.e. not because of any toxic effects) is at 12.3% - the relationship found is therefore not significant, and confidence intervals can not be calculated.

What do you do now?

The data basis can be increased. You can do that by adding more treatments - but that will in most cases mean you will have to repeat the whole experiment. If you have replicates available, you will be able to do it with the data you have: simply activate the option "Use Replicates while Fitting“ (see chapter 4.6.1). This will result in a significant dose response relationship (Figure 79). This option, however, is only available with metric data.

The way to get significant dose-response relationships with tight confidence intervals is to have a really large data basis! That means you will have to test as many concentrations as possible, or: fit them based on the replicates. The tested concentration range should cover the broadest possible span of inhibitions, as there should be no extrapolations from within the data range.

The parameter table contains two important measurements for the assessment of the received dose-response relationship: P(Chi²) (= unit of measure for the "goodness of fit") and P(F) (= unit of measure for the significance of the dose-response relationship). Please read the text for additional explanations.



Toxicity Metric	EC10	EC20	EC50
Value [µg/L]	0,0147	0,0177	0,0254
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

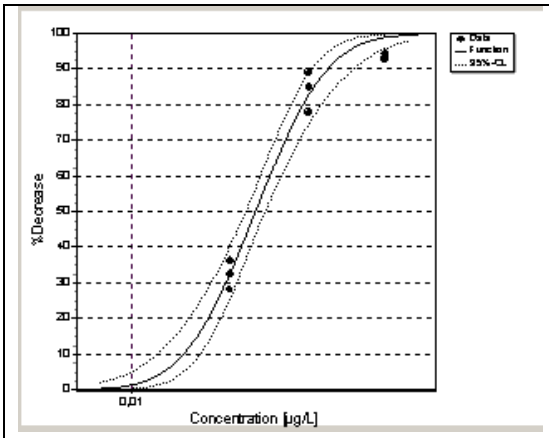
n.d.: not determined due to mathematical reasons or inappropriate data

Parameters of the probit a

Parameters of the probit analysis

Parameter	Value
Computation runs:	9,0000
Slope b:	5,3873
Intercept a:	8,5924
Variance of b:	52,3902
Goodness of Fit	
Chi²:	0,0213
legrees of freedom:	1,0000
p(Chi²):	0,8840
Log EC50:	-1,59494
SE Log EC50:	0,1671
g-Criterion:	6,2087
Variance (Chi²/df):	0,0213
r²:	0,9630
F:	26,0030
p(F) (df: 1;1):	0,1230

Figure 78: Example for non-significant dose-response relationship



Toxicity Metric	EC10	EC20	EC50
Value [µg/L]	0,0147	0,0177	0,0254
lower 95%-cl	0,0120	0,0153	0,0236
upper 95%-cl	0,0167	0,0196	0,0273

n.d.: not determined due to mathematical reasons or inappropriate data

Parameters of the probit analysis

Parameter	Value
Computation runs:	8,0000
Slope b:	5,3874
Intercept a:	8,5925
Variance of b:	17,4628
Goodness of Fit	
Chi²:	0,1259
egrees of freedom:	7,0000
p(Chi²):	1,0000
Log EC50:	-1,59494
SE Log EC50:	0,0965
g-Criterion:	0,0605
Variance (Chi²/df):	0,0180
r²:	0,9300
F:	92,4210
p(F) (df: 1;7):	<0.001

Figure 79: Example for significant dose-response relationship, achieved as a fit based on the replicates. Data from Figure 78.

6.3.4 Results Tables for ECx Determination, Non-Linear Regression

The following result outputs and/or result tables are generated for each linear regression:

- Overview of the inhibitions in percent
- Text: either a description of the calculated regression or information that the regression has been terminated without success (in this case the following tables are not displayed);
- Parameter table including significances (only available for Levenberg-Marquardt algorithm) and criteria for goodness of fit
- Analysis of Variance (analysis of the regression for significance) and test for „Lack of fit“
- Measurement values, predicted values and weighting factors
- Results: EC values and confidence range

If the regression was successful, ToxRat creates a description of the applied settings (Figure 80, upper part) and lists the result tables mentioned above. Important: "Successful" simply means that all steps of the regression were completed successfully, i.e. convergence has been achieved for the determined function parameters[4]. A successful regression does *not* necessarily mean that the result is significant and that the goodness of fit is sufficient! To assess this, you have to consider the parameter table, the results of test of significane, of the „Lack of Fit“ and of the remaining criteria for goodness of fit (see below). On the other hand, if ToxRat indicates, that there is "no convergence", this just points to the fact, that it might be promising to try other settings. It does not mean that the corresponding regression is not acceptable. If there is a significant dose response, the resulting ECx values look reasonable and the confidence limit are adequate, there is nothing to stop using the results. If no convergence could be achieved during regression, a corresponding output is created (Figure 80, lower part).

The 3-param. normal CDF F(x) = b0*[NormalCDF(b1-log10(x)/b2 + zOpt)] was fitted to the data (CDF: cumulative distribution function; b0-b2: parameters; zOpt: adjustment to have the EC10 as parameter b1; x: concentration).
An iteratively re-weighted non-linear regression with relative weighting (1/Y²) was performed.
The optimzation converged thus fitting was successful (Stop Reason = Converged (Optimization method: Levenberg-Marquardt)).

PLEASE NOTE: The non-linear regression procedure was terminated without achieving convergence due to mathematical problems (Stop Reason = Iterations > Max. Iterations (Optimization method: Levenberg-Marquardt)).
Please try whether an increase in the number of optimization cycles lead to convergence, or try modified settings, another function or another optimization method to get acceptable results.

Figure 80: Example output if the regression was successful (above) and if aborted (below)

[4] To say it simple: The derived function will be optimised in an iteration process until the changes of the parameters being achieved by this process reaches a predefined threshold value – i.e. until convergence is achieved.

The following section offers an overview of available criteria for goodness of fit (sorted by relevance). After that the different result tables where to find the criteria for goodness of fit are introduced and explained.

Criteria for goodness of fit for non-linear regression, sorted by importance

Analysis of Variance F Test	Checks if the dose-response relationship is significant	Objective: **Significance** (p(F) < 0,05); **mandatory**, otherwise EC values are of no relevance and no confidence limits are shown; the smaller p(F) is, the better the dose-response relationship
Lack of Fit, F Test	Checks if deviations between measurement values and function are simply by chance	Objective: **No significance** (p(F) > 0,05); (if so, then no „Lack of Fit“) not mandatory, but **desirable**; the greater p(F) is, the better the fit; maximum = 1
Function Parameters, t Test	Checks if the function parameters are significant	Objective: **Significance** (p(t) < 0,05); **advisable**; if one of the parameters is not significant, a function with less parameters should be selected
R^2 Coefficient of Determination	Specifies which part of the data variance is explained by the function	Objective: **high R^2 desirable. Attention:** other than for linear regression, R2 for non linear regression is difficult to interpretate and thus not suitable to select the best fit.
Akaike Criterion	Relative measure for goodness of fit, based on residual variance and number of parameters (amongst others)	**Decision-making aid**; if all other conditions are fulfilled (see above), select the function with the smallest AIC value.

The **Parameter Table** (Figure 81) contains the results for the function parameters. In case of the Levenberg-Marquardt algorithm, their standard errors and confidence intervals as well as their respective **significance** in the t test are also listed If not all parameter are significant, probably a function with too many parameters has been chosen. Try a function with less parameters in this case.
If it is a normal cumulative distribution function (CDF), one parameter represents the logarithm of one EC value. For more information see the table legend.

The value for the **coefficient of determination R^2** specifies, which part of the data variance is explained by the determined function. However – it is not such clear as with linear regression und should be regarded with care.
The residual standard deviation = square root of residual variance **(Residual Standard Error)** depends on the data scattering and has the same dimension as the data. So the residual standard deviation is relative to the measured data. Based on this value, you can

compare two different regressions with each other for the same data set. The smaller the residual variance, the better.
The **Akaike Criterion** (AIC) is a relative measure for goodness of fit. Its value is calculated from the residual variance, the number of parameters and the number of data. With the help of the AIC you can compare different non-linear regressions with individual settings for one and the same data set: the smaller the AIC value, the better the fit.
The **Shapiro-Wilks test** checks if the residues (i.e. the deviations of the observed values from the predicted function values) are normally distributed. If this is not the case (i.e. p(W) < alpha, recommended value for alpha = 0,01), the found function may deviate systematically from the observed data. This is important in the Bootstrap process, because normal distribution of residues is required. This criterion only informs you that the found function may not be optimal. If all other criteria for goodness of fit provide reasonable results, a significant Shapiro-Wilks test is tolerable.

Estimated parameters of the 3-param. normal CDF

Estimated parameters of the 3-param. normal CDF: Results of the non-linear regression analysis; b0 - b2: parameters; Std. Err.: standard error; 95%LCL|UCL: 95%-lower|upper confidence limits; t: t-statistic (Ho: b0|b1|b2 = 0); p(t): probability that the deviation from zero is due to chance (b1 = log EC10)

Parameter	Value	Std. Err.	95%LCL	95%UCL	t	p(t)
b0	50.575	1.011	48.555	52.596	50.035	<0.001
b1	0.185	0.071	0.044	0.326	2.622	0.005
b2	0.692	0.045	0.603	0.782	15.433	<0.001

Stop Reason = Converged (Optimization method: Levenberg-Marquardt)
R^2: 0.955; adjusted R^2: 0.953
Residual standard error: 0.40670
Akaike Criterion (AIC): 245.865
Shapiro Wilk`s test on normal distribution of residuals: p = 0.022.

Figure 81: Results of the non-linear regression: fit parameters and criteria for goodness of fit. Please read the text for additional explanations.

A reliable statement about the goodness of fit is provided by the analysis of variance and the test for „Lack of Fit“ (

Figure 82).
The **Analysis of Variance** checks if the received function explains a significant part of the variance. If this is not the case (i.e. p(F) > 0,05) the dose-response relationship is not significant. Therefore, the received EC values are of no relevance.The **F Test** checks the probability of obtaining a dose-response relationship from the sample data by chance - i.e. although in the basic population no dose response relationship exists. A significant dose-response relationship exists only if p(F) is smaller than 5%. Therefore, if p(F) is greater than 5%, the relationship found is non-significant. ToxRat will in this case issue a warning and no confidence intervals will be shown.
The test for „**Lack of Fit**“ checks, how well the function describes the data. It can be expected that the data points are not one hundred percent on the curve - the question is, however, whether the observed deviations are simply by chance, or if the selected function was incorrect. P(F) for Lack of Fit refers to the probability of the data points being chance

deviations. If p(F) > 5%, then there is no significant deviation between fit and data. The greater the value for p(F), the better the fit (max. value is 1 (=100%)). If you compare results from different functions for one data set, the following applies: The greater the value for p(F) for Lack of Fit, the better the data description of the function.

Analysis of Variance and Test for Lack of Fit for the 3-param. normal CDF

Analysis of Variance and Test for Lack of Fit for the 3-param. normal CDF: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p(F): probability that the variance explained by the regression is due to chance; Pure error: residual SS|MSS of an one-way ANOVA with the original data (CDF: cumulative distribution function)

Source	SS	df	MSS	F	p(F)
Regression	198,82	2	99,41	601,011	<0.001
Residuals	10,26	62	0,17		
- Lack of Fit	0,43	3	0,14	0,855	0,470
- Pure Error	9,83	59	0,17		
Total	208,25	64			

Since p(F|Regression) <= 0.05, a significant amount of variance is explained by the regression model.
Since p(F|Lack of Fit) > 0.05, there is no significant lack of fit.

Figure 82: Results of the non-linear regression: Analysis of Variance and Lack of Fit.

The next result table (Figure 83) compares the measurement values and the predicted values. In addition, here you will find the weighting factors used in the last iterative step, if weighted regression was conducted.

Observed and Predicted Results of the 3-param. normal CDF

Observed values in weight after 14,0 d as caused by the test item and predicted values as calculated from the function; Weight: weighting factors used in the final iteration step.

Treatm.[µg/L]	Observed	Mean Obs.	Predicted	Weight
0,010	50,000	50,0994	50,5755	0,0067
0,010	49,000	50,0994	50,5755	0,0067
0,010	52,100	50,0994	50,5755	0,0067
0,010	48,700	50,0994	50,5755	0,0067
0,010	53,000	50,0994	50,5755	0,0067
1,200	45,000	48,5170	46,7445	0,0078
1,200	48,000	48,5170	46,7445	0,0078

Figure 83: Results of the non-linear regression: Predicted Values and Weighting Factors

Finally, the calculated EC values with its confidence interval are output (Figure 84).

Point estimates from the 3-param. normal CDF

Point estimates from the 3-param. normal CDF: Selected effective concentrations (ECx) of the test item; cl: confidence limit

Toxicity Metric	EC10	EC20	EC50
Value [µg/L]	1,531	3,086	11,807
lower 95%-cl	1,106	2,237	7,946
upper 95%-cl	2,118	4,262	17,399

n.d.: not determined due to mathematical reasons

The confidence limits of the EC10 used as a parameter were computed by means of the standard error of parameter b1; confidence limits for the remaining ECx were estimated by Monte-Carlo simulation

Figure 84: Results of the non-linear regression: ECx Values and Confidence Limits

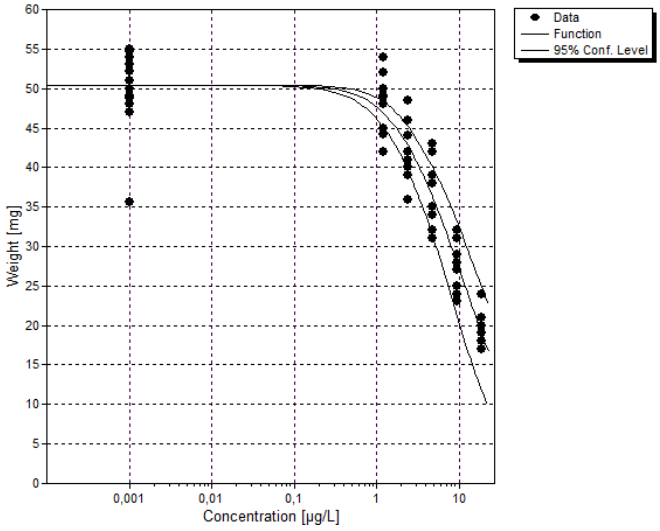


Figure 85: Results of the non-linear regression: graphical output. Original data are shown.

6.3.5 Result Tables for Parametric Tests - MDD as a Measure for Statistical Power

ToxRat will determine the minimum detectable difference, (MDD) for all parametric tests (t test, Dunnett, Williams) (Figure 86). The MDD tells you what the minimum response (i.e. the minimum percentage difference between the control value and the treatment value) must be in order to be detected as statistically significant in the current test.
MDD is therefore a measure of the statistical power - the smaller the MDD, the more powerful the test. The MDD depends on sample size, variance and the selected statistical test. The larger the sample size (= number of replicates) and the smaller the variance, the smaller (i.e. better) the MDD. One and the same data set will result in different MDDs depending on the statistical test used (see also Figure 21).

MDD is sort of a "detection limit" for statistical tests. It will allow you to assess how meaningful a test result of "significant" or "non-significant" really is. You should therefore pay particular attention to the MDD column in the relevant tables.

STUDENT-t test for Homogeneous Variances with Bonferroni-Holm Adjustment'

STUDENT-t test for homogeneous variances with Bonferroni-Holm adjustment with weight at 14,0
d: Multiple sequentially rejective comparisons of treatments with "Control". Significance was
Alpha = 0,05, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation;
MDD: minimum detectable difference to Control (in percent of Control); 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(t) <= 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	df	%MDD	t	p(t)	Sign.	Sign.
Control	50,10	3,850						
1,200	48,52	3,850	59	-5,06	-1,04	0,151	0,050	-
2,400	41,82	3,850	59	-6,28	-5,27	< 0.001	0,025	+
4,800	36,30	3,850	59	-6,6	-9,09	< 0.001	0,017	+
9,600	27,30	3,850	59	-6,97	-15,02	< 0.001	0,013	+
19,200	19,50	3,850	59	-7,81	-18,7	< 0.001	0,010	+

+: significant; -: non-significant
A NOEC of 1,200 µg/L is suggested by the program.

Figure 86: Result table for parametric test including the MDD as unit of measure for test strength (in this case: 5.06% - 7.81% - i.e. a very strong test).

6.4 Formatting

6.4.1 Decimal Separator

You cannot specify the type of decimal separator to be used in ToxRat (comma or full stop), because ToxRat will simply apply the relevant system settings of the operating system (WIN XP: System Settings >> Regional and Language Options; WIN7: System Settings >> Time, Language, and Region). Should you need the ToxRat tables or reports to use a different decimal separator than the one set as default on your PC, then you will have to change the system settings before you open ToxRat.

6.4.2 Number of Decimal Digits

You will find the relevant formatting settings at the following locations in the program depending on the type of values (test concentrations, inhibition values, key indicators like mean values, end points like ECx and NOEC, ...):

Generic Workbooks

Sheet Generic Notes	Figure 87
Row 21 (Decimals data)	The number of decimal digits for statistical **basic key indicators**, including mean value, standard deviation, median, minimum, maximum, etc.
Row 22 (Decimals concentrations)	Number of decimal digits for a) **ECx and NOEC values**, and b) the **test concentrations** entered into the InputRawData sheets. The result is a unified number of decimal digits for test concentrations in data and results tables, with zeroes added where necessary. Important: For the value set here to take effect, "use formatted values" must be selected in Options >> Input/Output (Figure 88, is the default value).
Row 23 (Decimals time periods)	Number of decimal digits for the **measuring time(s).**
Menu Options >> Input/Output	Figure 88
Test concentrations	If the test concentrations are to be displayed the way they are input into the data input sheet (i.e. with different numbers of decimal digits), then simply deselect "use formatted values".
Percentage values	Number of decimal digits for **percentages**, e.g. % inhibition, % MDD, % mortality
Probabilities	Number of decimal digits for **statistical probabilities**, p(test value).

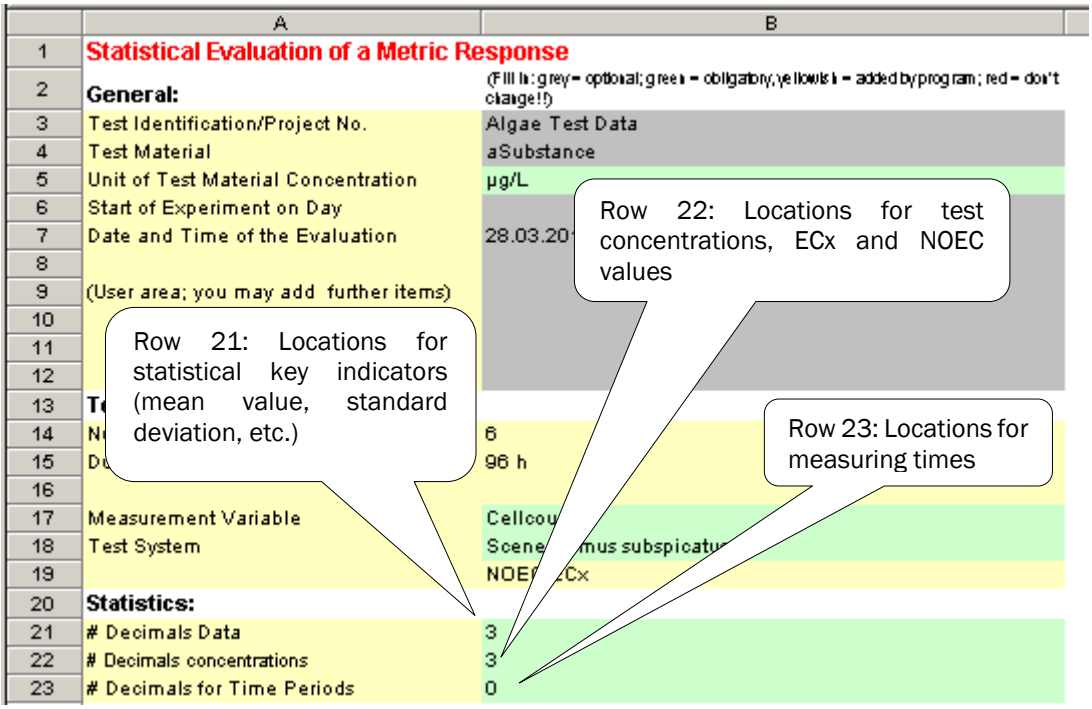


Figure 87: Formatting settings for Generic Workbooks, sheet "Generic Notes"; for Biotest Workbooks row 21 is not needed, instead, see Figure 88.

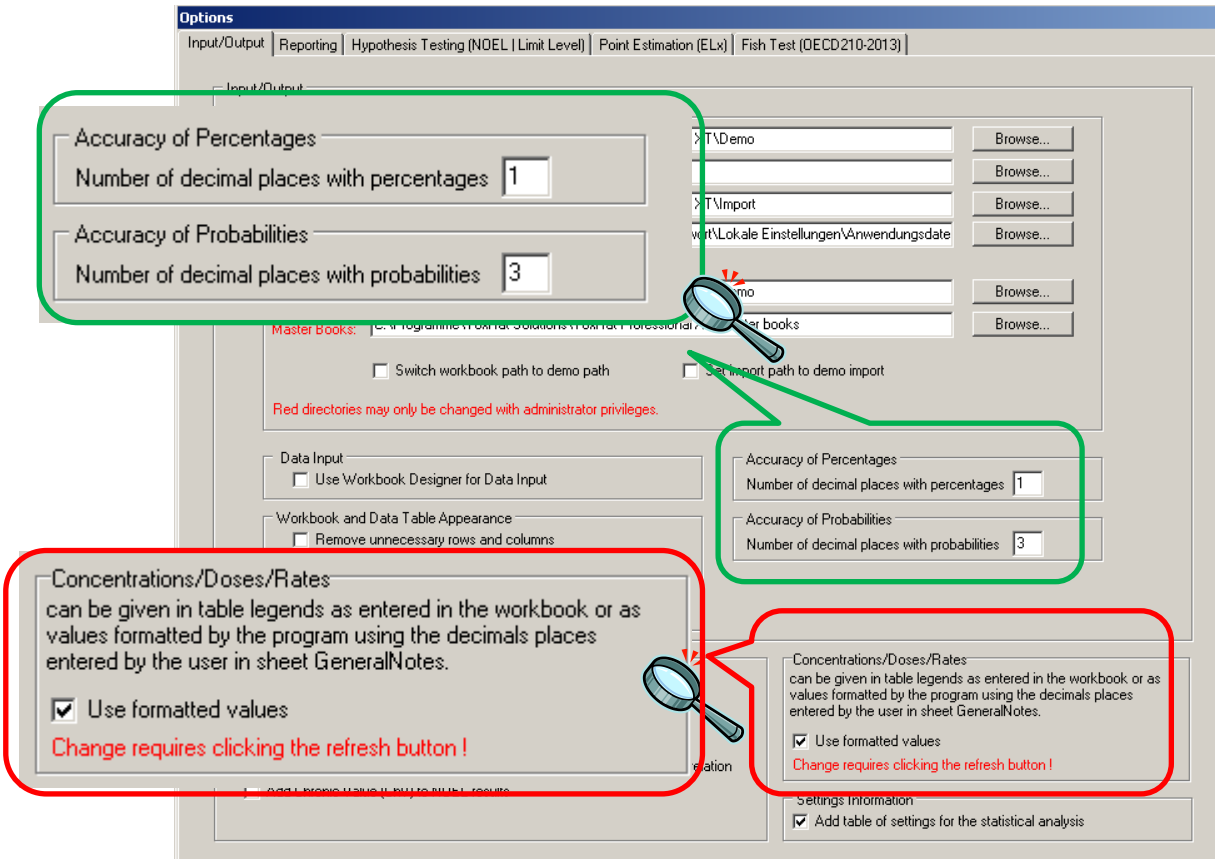


Figure 88: Formatting settings in the dialogue Options >> Input/Output

Biotest Workbooks

The rules for formatting settings are generally the same for Biotest Workbooks as for Generic Workbooks (see above, Figure 87 and Figure 88) – with one exception:
Since there may be more than one variable in a Biotest Workbook, the number of decimal digits for **statistical key indicators**, like mean value, standard deviation, median, minimum, maximum, etc. is not set in row 21 of the sheet "Generic Notes", but can be set individually for each value in the dialogue Options NOEC below the green field for variable selection ("decimals of current variables in tables") (Figure 89).

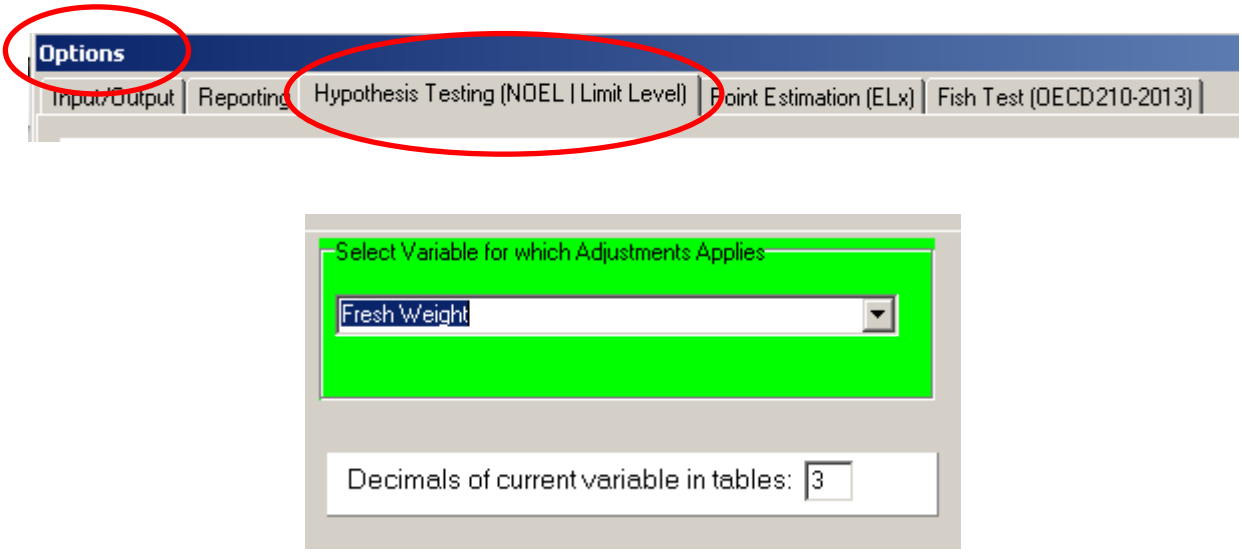


Figure 89: Variable-specific settings for decimal digits of statistical key indicators (mean value, standard deviation, median, minimum, maximum, etc.) in Biotest Workbooks.

Decimals digits for parameters of non-linear regression
Regardless of which workbook type you are using, the number of decimal digits for parameters of non-linear regression are set in the menu “Non-linear Regression” (Find Effect level – Non Linear regression – Further Options; Box “Presentations”, see Figure 36).

The following applies no matter what the number of decimal digits selected in the tables: ToxRat will always include all calculated decimal digits in its calculations! This may result in slightly different results if you choose to recalculate some evaluations manually, and use rounded values from the data tables for your calculations.

6.5 Optional Settings

Or: What settings do you really want?

In this section we will talk about various optional settings for results presentation in ToxRat.

6.5.1 Chronic Value (ChV)

Some US guidelines (EPA/OPPT, Environmental Protection Agency / Office of Pollution Prevention and Toxics) require the so-called "chronic value" as a further toxicity parameter (ChV) in addition to NOEC and ELx. ChV is defined as the geometrical average of the “no observed effect concentration” (NOEC) and the “lowest observed effect concentration” (LOEC), and is calculated as ChV = 10^([log (LOEC x NOEC)]/2).
ToxRat will output this additional parameter if you select "Add Chronic Value (ChV) to NOEL results“ (Figure 90 on the left), under Options >> Input/Output. By default, this setting is deactivated.

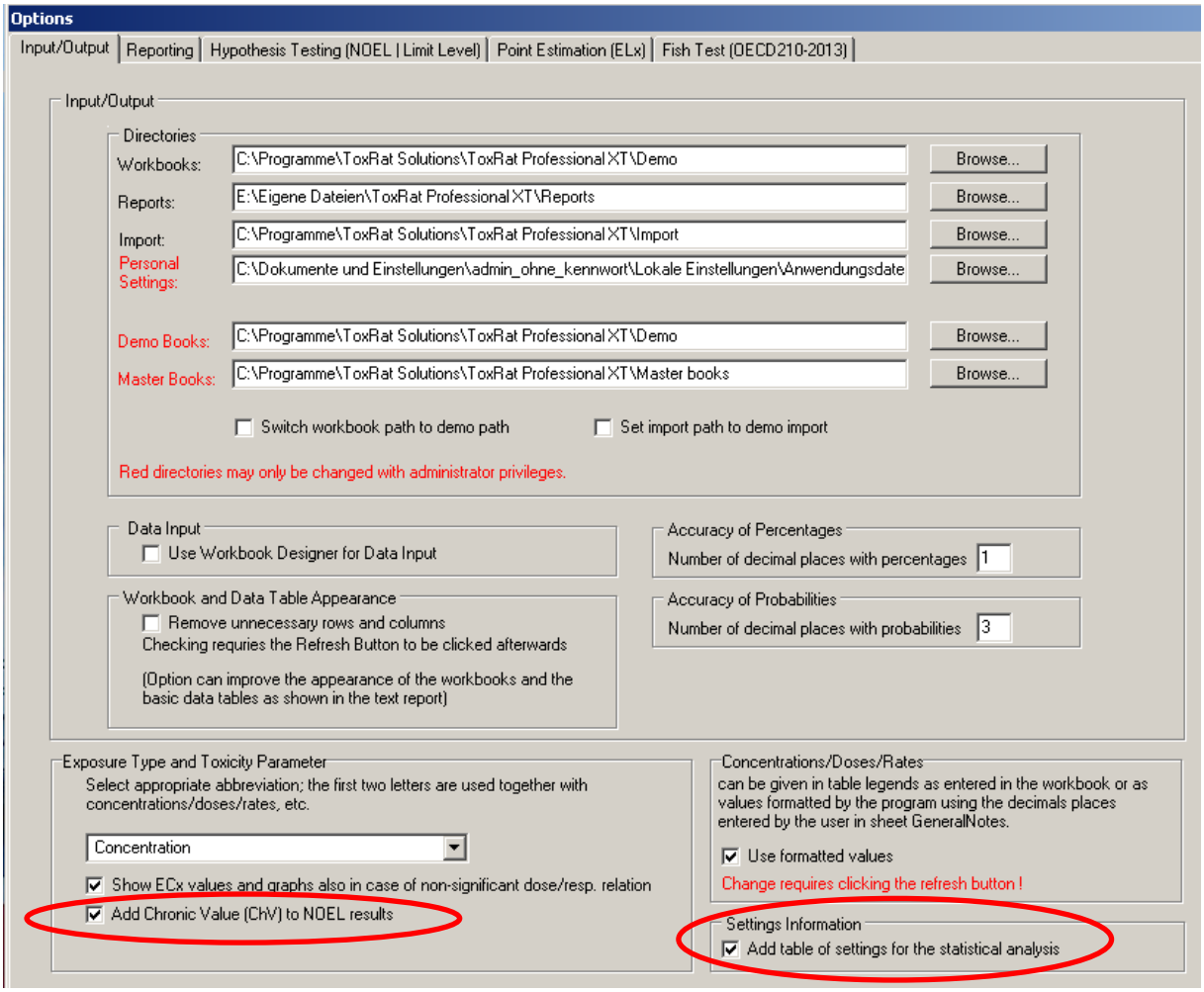


Figure 90: Setting option for the output of the chronic value (ChV) on the left, and for an additional settings table on the right

6.5.2 Settings

ToxRat will generate a so-called settings table to support the reproduction especially of those evaluations that were not carried out using the default software settings, but with user-defined settings instead. The table documents all settings used for the current evaluation, and the ones differing from the software default settings can be identified (Figure 91). By default, the settings table will be shown last (after "Summary"), unless you unselect "Add Table of Settings for the Statistical Analysis" (Figure 90 on the right) under Options >> Input/Output. It will also be listed on the report, unless you have deselected the relevant element in the report menu (see chapter 7).

Fish, Early-life Stage Toxicity Test (OECD 210-2013): aProject

Settings Table

Area	Item	Default Settings	User Settings
Global			
	Type of Exposure		Concentration
	Extrapolation of ECx	By program	By program
	Show non-significant ECx	YES	YES
	Statistical design	NOEC/ECx	NOEC/ECx
Variables			
Hatchability	State	Selected for analysis	Selected for analysis
	Data transformation	none	none
	Decimals data	1	1
	Final testing		
	Test procedure	SD Cochran-Armitage	Bonferroni Fisher
	Who selected final test	Program	User
	Additional tests	None	None
	Significance level	0,05	0,05
	Test direction	one-sided greater	one-sided greater
	LCx computation		
	Selected LCx values	LC10, LC20, LC50	LC20, LC30, LC50, LC90
	Selected method	Linear Regression	Linear Regression
	Regress. type	Max. Likelihood	Max. Likelihood
	Dose/response function	Probit (normal sigmoid)	Probit (normal sigmoid)
	Sig. level goodness of fit	0,10	0,10
	Data	Treatment mean/total	Treatment totals
	Confidence limits	after Fieller	after Fieller
	Control mortality	Not compensated	Compensated after Abbott
Post-hatch survival	State	Selected for analysis	Selected for analysis
	Data transformation	none	none
	Decimals data	0	0
	Final testing		
	Test procedure	SD Cochran-Armitage	SD Cochran-Armitage

Figure 91: The settings table (extract) offers an overview of the settings selected for an evaluation (example: Workbook OECD 210)

6.5.3 Output of Non-significant and/or All EC Values

It might be that - depending on the data situation - EC values can formally be calculated, but the received dose-response relationship is non-significant (see chapter 6.3.3). ToxRat will in this case issue a warning and will not calculate the confidence intervals. The dose-response functions will, however, be mapped in a graph, and any ECx values values derived will be output to allow you an overview of the actual data situation.
Alternatively you could select settings to suppress the generation of graphs and the display of EC values for non-significant dose-response relationships. Simply unselect "Show ECx values and graphs also in case of non-significant dose-resp. relation." (Figure 92).

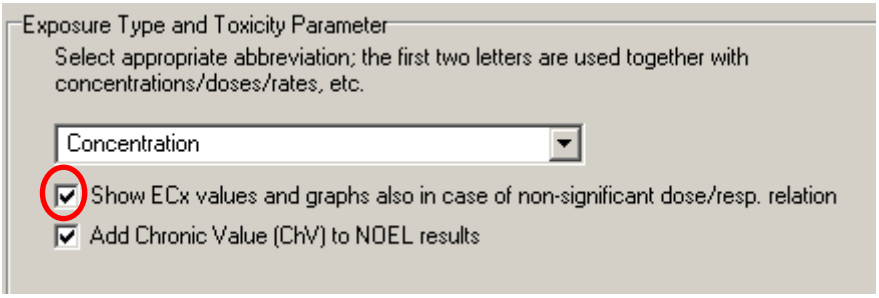


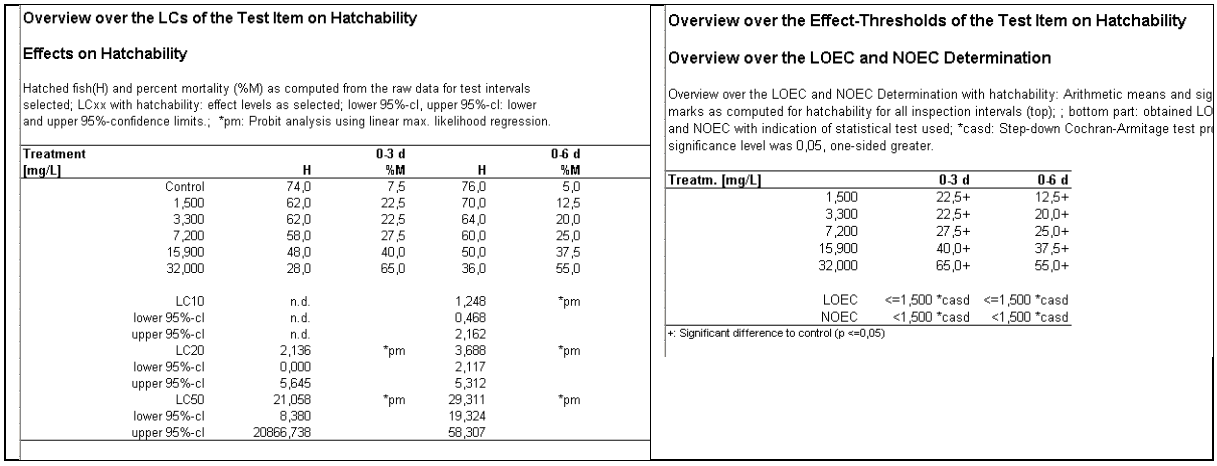
Figure 92: Go to Options >> Input/Output to select setting options for handling non-significant dose-response relationships

In addition to the up to 6 selectable effect levels for which EC values and confidence intervals are output (chapter 4.6 and Figure 31), ToxRat can also output the entire value table for the calculated dose-response function from 0.1% response to 99.9% response, including the upper and lower confidence limits. Simply select "Add table with all ECx-values" under Options >> Report (Figure 94). Important: Since the generated tables are very large, they will not be displayed in the result sheets, and can instead be selected under the menu item "Tables" under the name "<variable name> ConcResp Function <measuring time>" for display in a separate window. They will be automatically integrated in the report. This option cannot be set for individual variables, but will instead apply for all variables within a workbook.

6.5.4 Generating Overview Tables

Overview tables are available for the results of the dose-response analysis and for the results of the NOEC find effect level (see Figure 93 for examples) – you can specify when and if ToxRat should display them (Options >> Reporting, Figure 94):
- only if more than one measuring time is evaluated (default setting)
- always, even if only one measuring time is available
- never

The setting will apply for the result data sheets as well as for the report.



Overview over the LCs of the Test Item on Hatchability

Effects on Hatchability

Hatched fish(H) and percent mortality (%M) as computed from the raw data for test intervals selected; LCxx with hatchability: 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 [mg/L]		0-3 d H	0-3 d %M	0-6 d H	0-6 d %M
	Control	74,0	7,5	76,0	5,0
	1,500	62,0	22,5	70,0	12,5
	3,300	62,0	22,5	64,0	20,0
	7,200	58,0	27,5	60,0	25,0
	15,900	48,0	40,0	50,0	37,5
	32,000	28,0	65,0	36,0	55,0
	LC10	n.d.		1,248	*pm
	lower 95%-cl	n.d.		0,468	
	upper 95%-cl	n.d.		2,162	
	LC20	2,136	*pm	3,688	*pm
	lower 95%-cl	0,000		2,117	
	upper 95%-cl	5,645		5,312	
	LC50	21,058	*pm	29,311	*pm
	lower 95%-cl	8,380		19,324	
	upper 95%-cl	20866,738		58,307	

Overview over the Effect-Thresholds of the Test Item on Hatchability

Overview over the LOEC and NOEC Determination

Overview over the LOEC and NOEC Determination with hatchability: Arithmetic means and sig marks as computed for hatchability for all inspection intervals (top); ; bottom part: obtained LO and NOEC with indication of statistical test used; *casd: Step-down Cochran-Armitage test pr significance level was 0,05, one-sided greater.

Treatm. [mg/L]	0-3 d	0-6 d
1,500	22,5+	12,5+
3,300	22,5+	20,0+
7,200	27,5+	25,0+
15,900	40,0+	37,5+
32,000	65,0+	55,0+
LOEC	<=1,500 *casd	<=1,500 *casd
NOEC	<1,500 *casd	<1,500 *casd

+: Significant difference to control (p <=0,05)

Figure 93: Structure of overview tables to find effect levels (left) and for determining NOEC values (right).

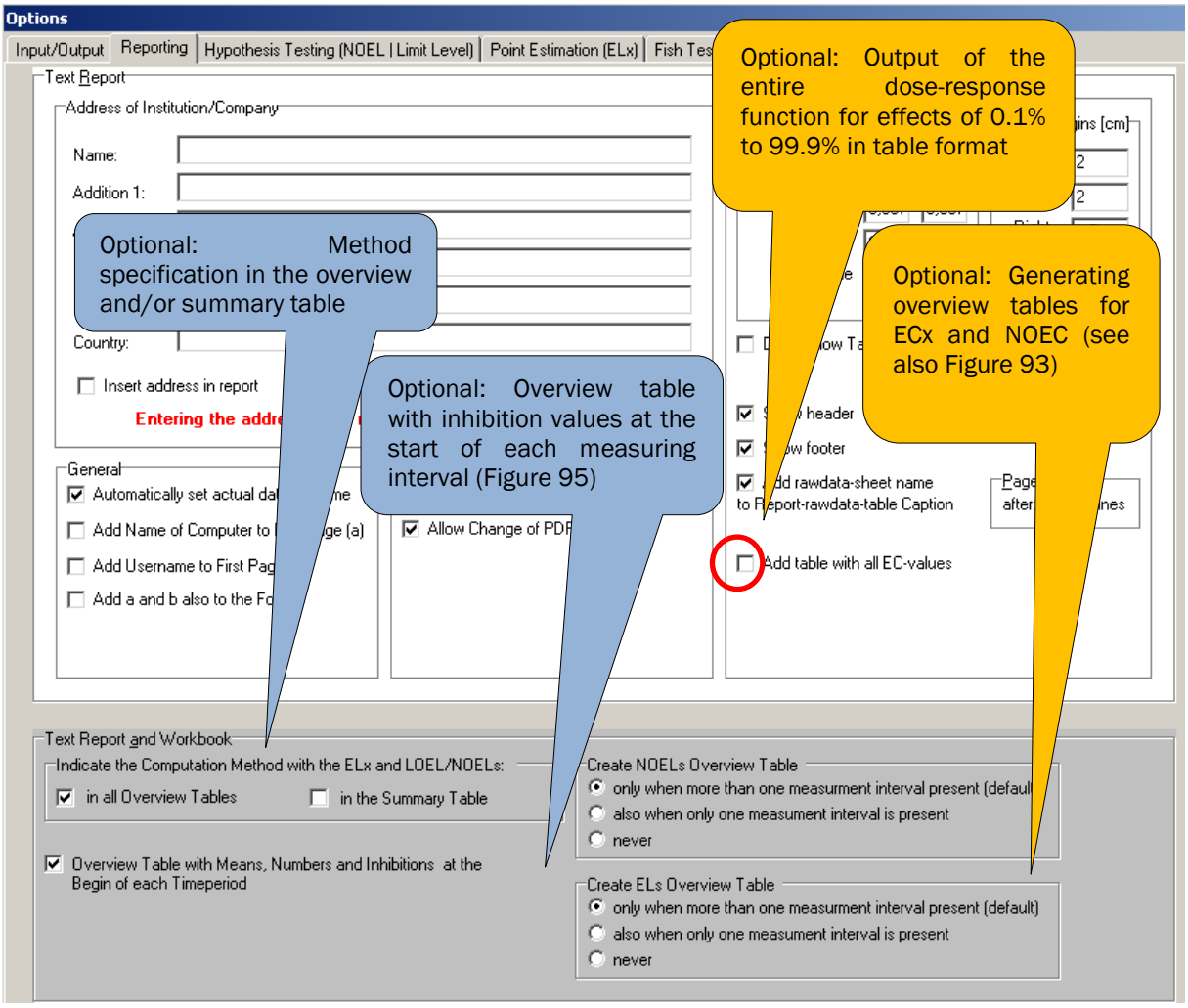


Figure 94: Optional setting for the result output in the selection dialogue Options >> Report. Default program settings shown.

Select under Options >> Reporting, whether the generated overview and summary tables should also include the relevant statistical methods used (in the form of abbreviations, which are explained in the legend) (Figure 94). This is a default setting for overview tables, but not for the summary table. The setting selected here will be applied to all variables in the workbook.

ToxRat will by default generate an overview table with the measured mean values and inhibition values at the start of each measuring time (Figure 95). You can deselect that function under Options >> Reporting (Figure 94).

Length in Danio rerio after 35 d.

%Reduction of length caused by the test item after 35 d.

Treatm.[mg/L]	Mean	Std. Dev.	n	%Reduction
Control	12,34	0,993	4	
1,500	11,02	0,768	4	10,7
3,300	10,72	0,208	4	13,1
7,200	10,53	0,202	4	14,7
15,900	9,95	0,173	4	19,4
32,000	7,75	0,289	4	37,2

Figure 95: Example of an optional overview table with mean values, sample sizes, and inhibition values displayed at the start of each measuring time (system default setting).

6.5.5 Automatical Documentation of Date and Time of Evaluation

With default settings, ToxRat automatically sets date and time of the current evaluation into cell B7 of sheet General Notes and hence also into the report. You can deselect this function under Options >> Reporting (Figure 96). If the checkbox is deselected, the current content of cell B7 is used in the report (i.e. either empty cell or user definded entry).

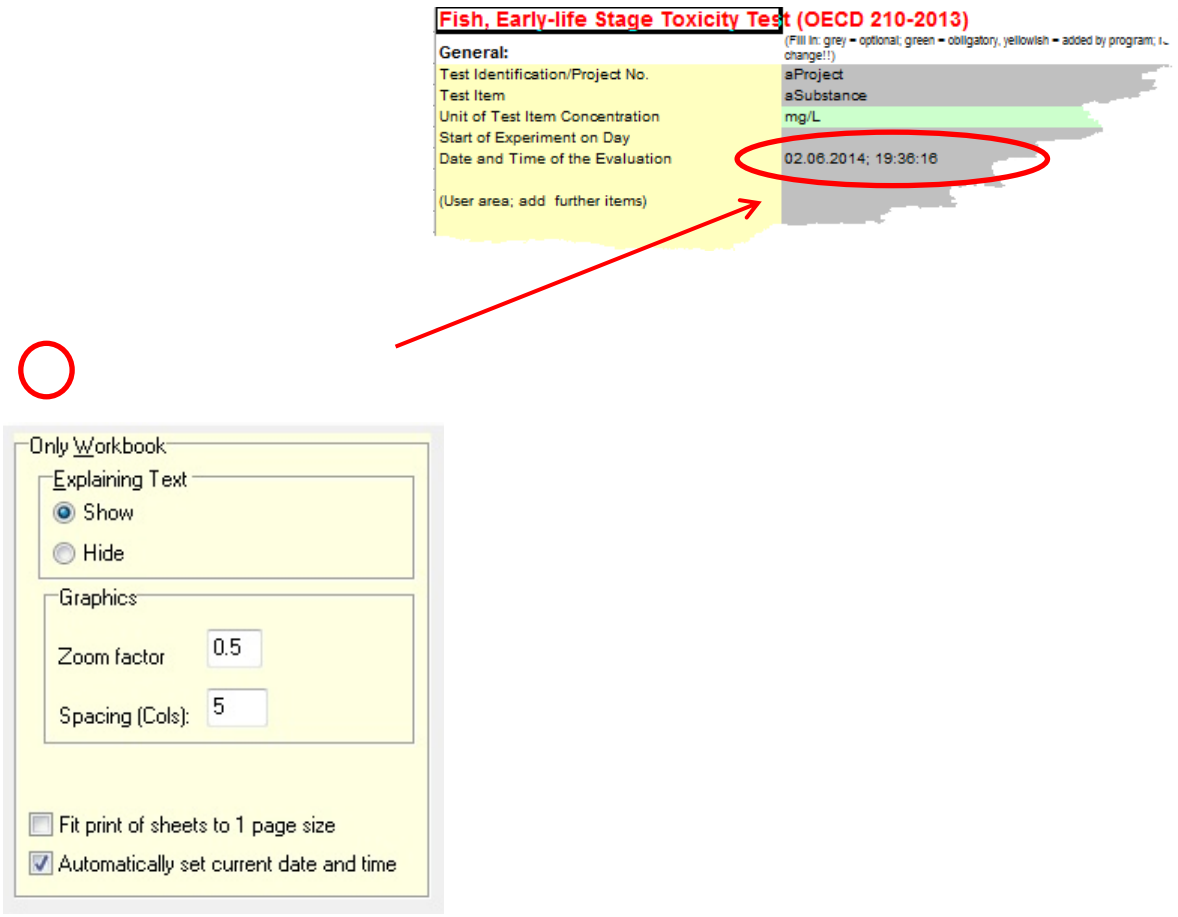


Figure 96: Menu Options – Reporting: Selection of automatical Setting of date and time of evaluation; default setting: selected

7 The Report

Or: How to visualise your results

With ToxRat, your route from the results to a full report entails nothing more than the click of a button. Here too, default settings are provided for you. For reasons of GLP,[5] reports can only be created from the results of an evaluation carried out just beforehand. That is why you can only find the report generation command in the result dialogue: once in the menu bar (Figure 97) and once in the graph window (Figure 98).

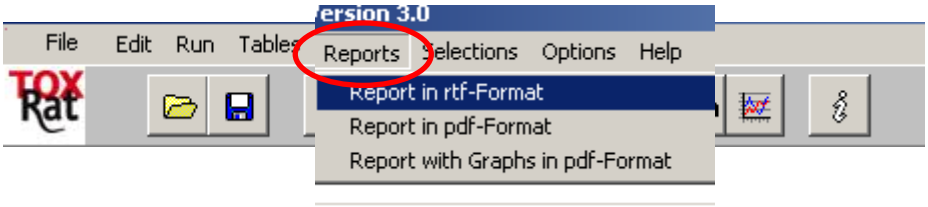


Figure 97: Commands for report generation in the menu bar of the results screen

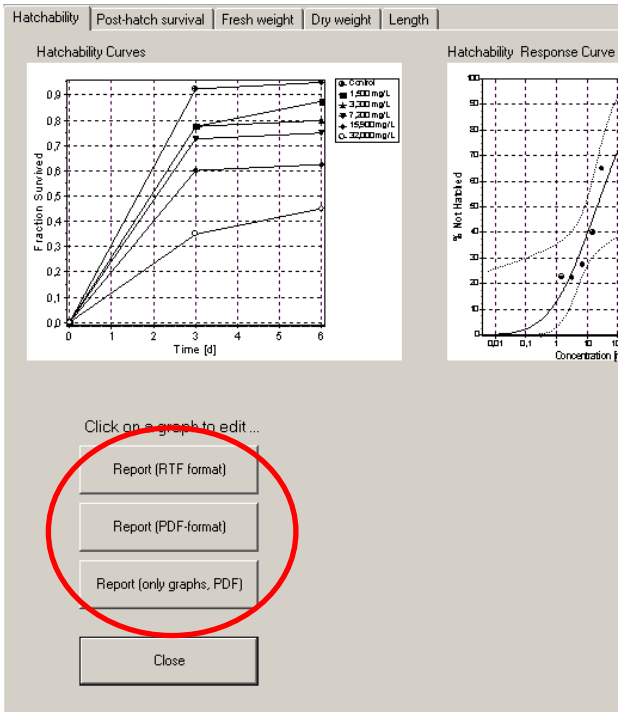


Figure 98: Commands for report generation in the graph window

[5] GLP = Good Laboratory Praxis

There are three report variations available:
- full report in rtf format
- full report in pdf format
- graphs only in pdf format

There are two distinctive differences between the rtf and pdf report formats:

1. The rtf report generated in ToxRat shows a black frame around all text pages - this sets it visually apart and signals that it is a primary report generated directly from an evaluation without any editing. The primary report can be printed directly from within ToxRat. But don't forget: ToxRat is NOT a text editor! The formatting, page breaks, etc. of the primary report may not always be perfect.
2. The generated rtf report can be saved and then opened in a text editor, where you can correct the formatting and can adapt the report to whatever templates you use.

The rtf report generated by ToxRat can be easily identified as an unedited primary report by its black frame. It can be printed and archived with the frame. Once the rtf report has been saved as a file and reopened, the black frame will disappear. This will identify the report as a secondary report, in which the content may have been altered.

You can select the elements you wish to include in the report dialogue before it is generated by ToxRat. This and other functions that will help with report creation are explained in the following section.

7.1 Menus and Buttons in the Report Screen

Selecting an rtf or pdf report will open a dialogue with specific setting options for report creation (Figure 99) (exception: pdf report containing only graphs; there will be no element selection available, and the report will be generated immediately).
On the left side of the report dialogue, you will see a list of all raw data and result tables created in the evaluation, as well as graphs, summaries, and possibly the result of the validity check - all with their relevant main and sub-headers. All elements you select here will be included in the report - if you make no specific selection, then all elements will be included by default. Clicking the button "Generate Report" will initiate the report creation.

Simply remove the check mark next to any elements you don't want to include in the report. To make things simple, we have included the functions "Select all Items" and "Clear Item List" - these allow you to either select or deselect all elements in one go.
The list of available report elements is very long for extensive biotests, and it can be quite tedious to keep looking for specific elements that you want included or excluded. That is why we have the so-called report profiles:
You can save a particular report profile ("Save report settings") if you have a specific set of elements with which reports have to be repeatedly created. The next time you evaluate a biotest with the same criteria, you can simply reuse your stored report profile ("Use stored settings"). Up to five different report profiles can be stored for each biotest.

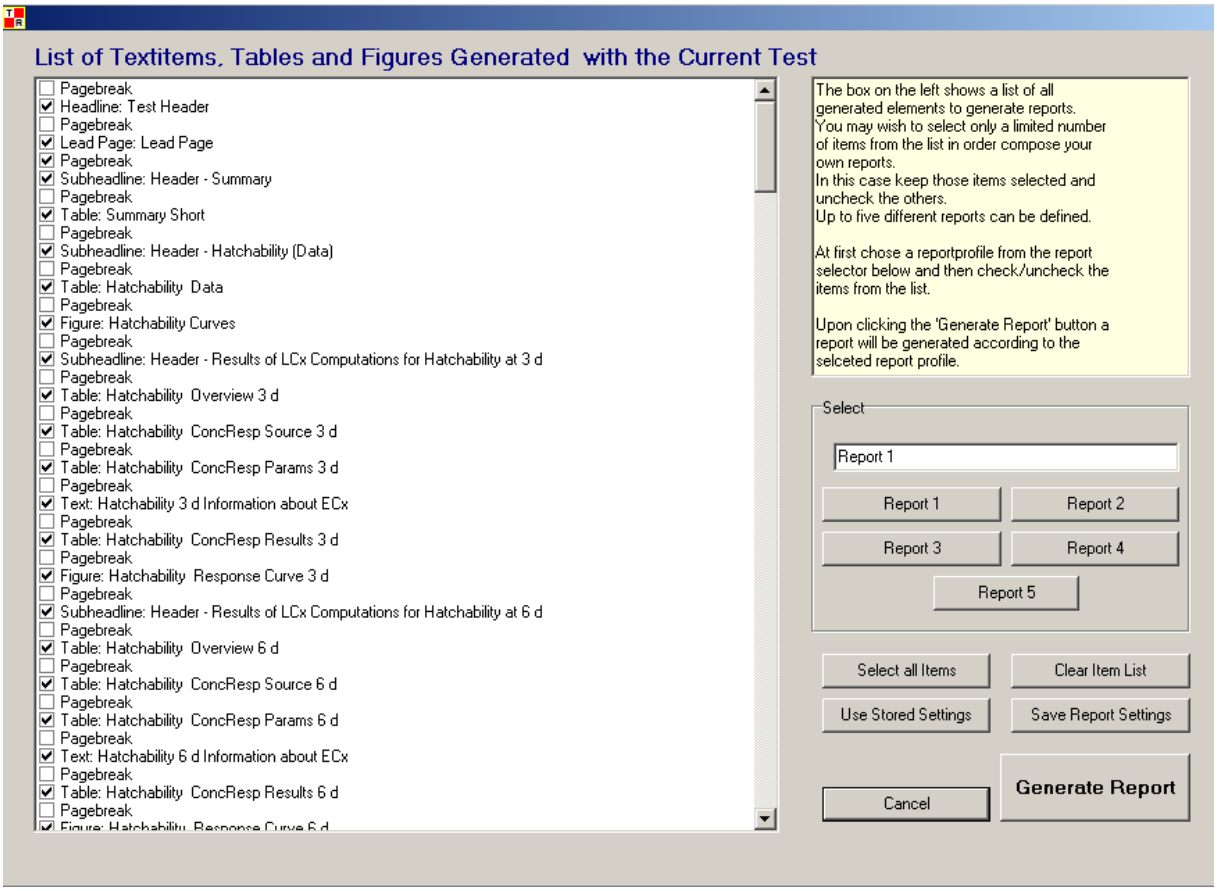
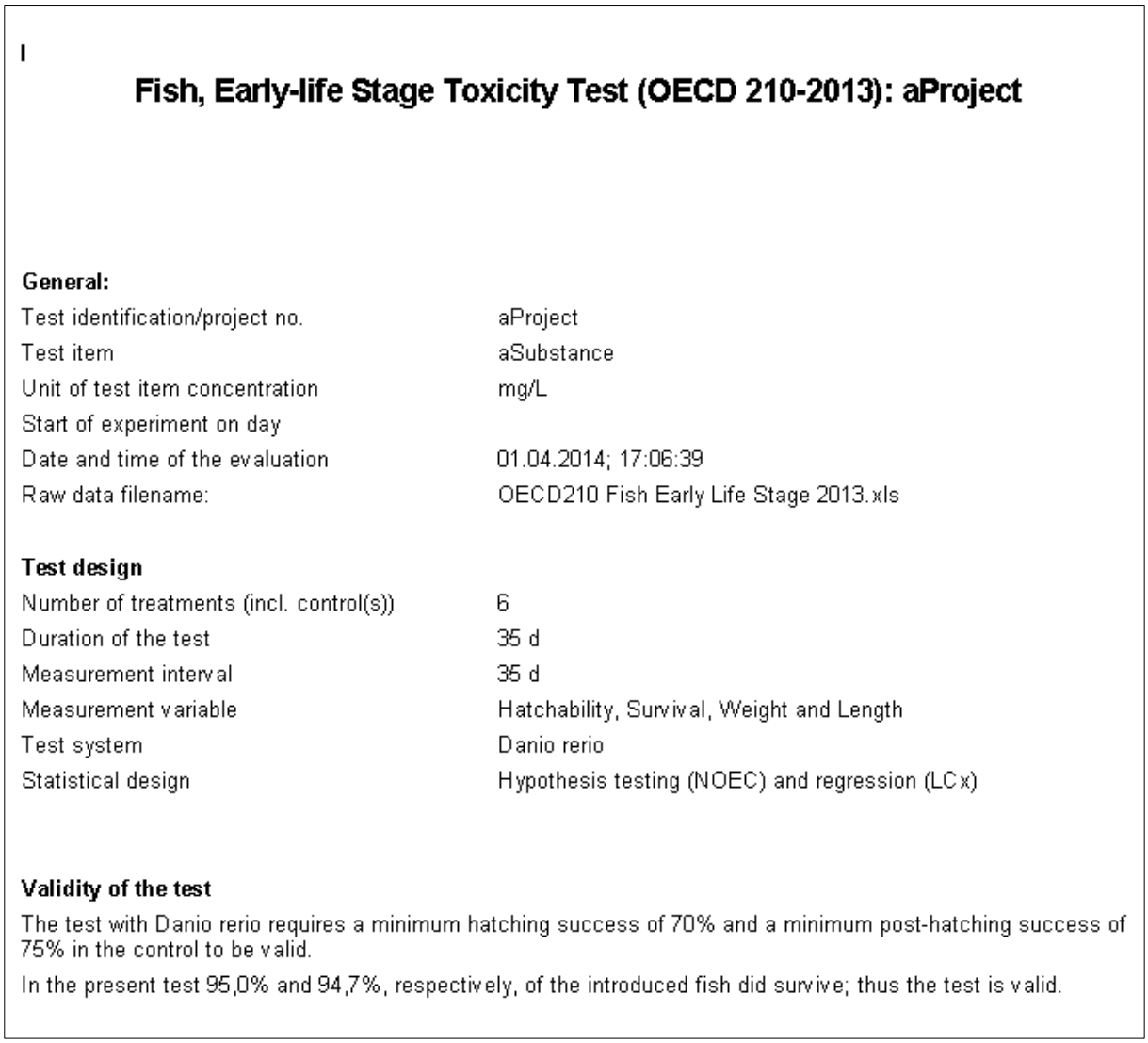


Figure 99: Dialogue with setting options for report creation.

When creating a pdf report, ToxRat will prompt you for a directory where to save it (path). Once saved, the pdf file will be automatically opened for viewing (unless you have deactivated the relevant function – see section 7.2). A newly created rtf report will be displayed in a new window. You can then choose to print or save the report via the "File" menu of the report window.

7.2 Optional Report Settings

There are default settings available that will allow you to generate a report at any time immediately, simply by clicking "Generate Report". The report will by default begin with the so-called title page, which contains the information from the data sheet General Notes regarding the test name, substance, etc. The result of the validity check can be found directly underneath (Figure 100).



Fish, Early-life Stage Toxicity Test (OECD 210-2013): aProject

General:

Test identification/project no.	aProject
Test item	aSubstance
Unit of test item concentration	mg/L
Start of experiment on day	
Date and time of the evaluation	01.04.2014; 17:06:39
Raw data filename:	OECD210 Fish Early Life Stage 2013.xls

Test design

Number of treatments (incl. control(s))	6
Duration of the test	35 d
Measurement interval	35 d
Measurement variable	Hatchability, Survival, Weight and Length
Test system	Danio rerio
Statistical design	Hypothesis testing (NOEC) and regression (LCx)

Validity of the test

The test with Danio rerio requires a minimum hatching success of 70% and a minimum post-hatching success of 75% in the control to be valid.

In the present test 95,0% and 94,7%, respectively, of the introduced fish did survive; thus the test is valid.

Figure 100: Report title page generated with default settings

Advanced users can check the menu item "Options - Reporting" for optional report settings (Figure 101).

Address

You can enter your address information here if you like. It will then be printed at the top of the report title page (select "Insert Address in Report"). The input or modification of address data will require one-time administrator rights.

Summary

The report will begin with a short summary as standard, i.e. an overview of all results *for the last measuring time.* A complete summary, including all intermediate measuring times, can be found at the end of the report. Select the check box "Start report with full summary" if you would like to see the complete summary first.

Information within the Scope of GLP

ToxRat can display the name of the computer or the name of the current user on the title page or in the footer of the report; you simply have to select the relevant options.

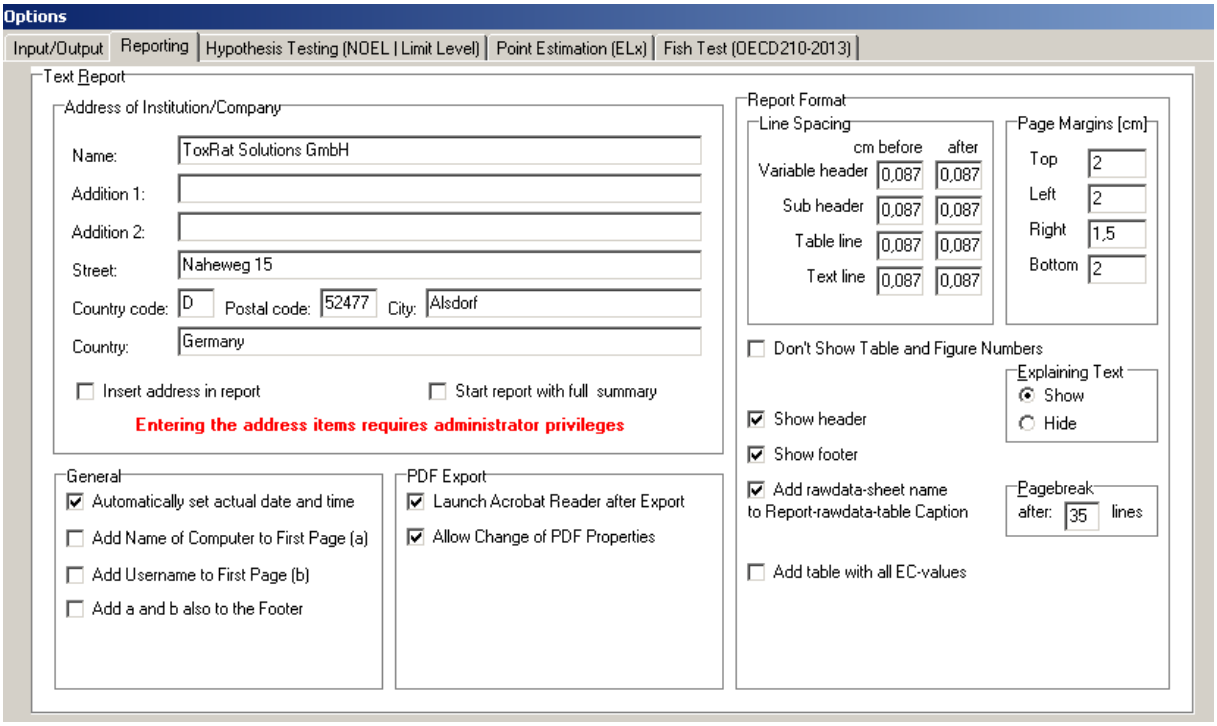


Figure 101: Menu for optional settings for report creation; shown here: Default settings

8 GLP Conformity and Validation

The following ToxRat attributes help with GLP requirement compliance:

Unchangeable
All data templates can be filled out and edited directly in ToxRat (integrated data input tool; see chapters 3.4 and 3.5 for more information.). MS Excel will not be required. All of the evaluations will be carried out exclusively by ToxRat internally and without any need for MS Excel functions. In other words: ToxRat can be run on any computer with Windows OS, no matter if any other program - and specifically MS Excel - is a) installed at all, and b) available in a particular version.

The primary report can, as a rule, only be generated on the basis of *an evaluation that has just been completed*, i.e. it will always be created from an internal original copy of the results. Any manual changes to the results implemented during the course of a program session will not be applied. It is therefore *impossible* to amend result tables before they have been applied to the report. The primary report can be saved, and - if you have selected the rtf format - it can of course also be edited. The primary report, which is printed out directly, can be easily distinguished from an archived (and possibly edited) report. The report will contain any information you would like to include: the ToxRat version used, the raw data file name, the evaluation date, computer name, username, and more (for more details see chapter 7).

Verifiable
The algorithms and statistical processes used in ToxRat are certified. The scope of delivery includes a validation document explaining all methods and processes (including the mathematical formulae on which they are based) used by the program. All mathematical and statistical procedures are applied to standardised test data sets, and the results are verified in comparisons of the results with independent calculations (e.g. MS Excel) and data published in literature.

Repeatable
The test data sets form part of the scope of delivery of the software, which means that you, the user, can repeat this quality control step yourself, and can verify that the software delivers the correct results stated in the validation document whatever your individual system configuration.

Please read the validation documentation for more information on the topic.

9 Installation

9.1 Local Installation

Run the .exe file.
You will need full administrator rights during the installation process and when opening the program for the first time, at which time you will have to register. Important: Make sure to initialise the program the first time with the username that is to be used for later program uses.
Please note also, that WIN7 seperates between "usual admin rights" and "advanced admin rights" - with the latter not available (and might be even not visible) by default. So, if the program closes without siplaying the registration window, you possibly have to activate the special administrator profile for the first launch of ToxRat.

If the error message “You need full administrator privilegues to start ToxRat“ is displayed: ToxRat does not accept domain accounts with local admin rights as admin users. The installation will complete without errors, but the above error message will be displayed at first initialisation, and the program will close. Solution: Create a local user on the relevant PC and add that user to the local admin group.

Some system files required for running the software will be written to the local Windows system directory. Each program variant (e.g. Standard, Monitor, Professional, or Professional XT) will be installed in an own program directory. The following paths apply for installations under WIN7.

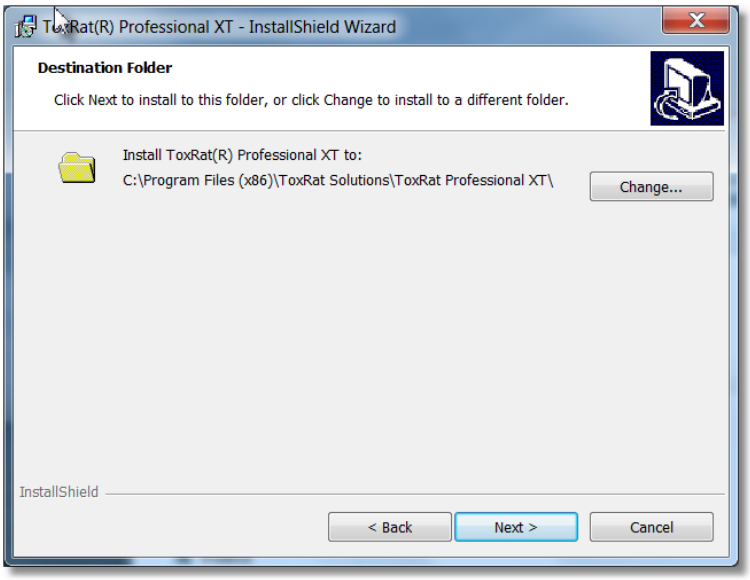


Figure 102: Default program directory for installations under WIN7.

You will find ToxRat.exe and the files and folders shown in Figure 103 in the program directory, once installation has been completed successfully.
Please note: in order to minimize the file size, the installation files do not include any manual. The manual is available both in eglish and in german via download from the ToxRat website.

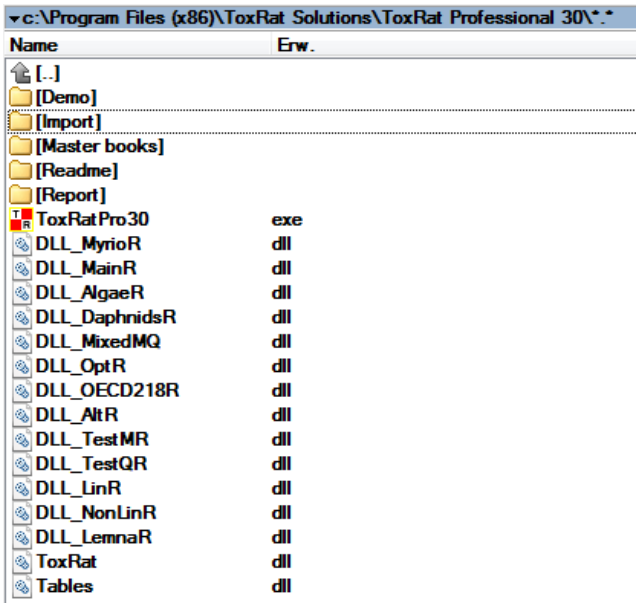


Figure 103: File structure created automatically in the program directory during installation.

The file **ToxRat.dll** will be copied to a user-specific directory (see below) during the first program initialisation. Its purpose is to store the following user-specific settings:
a) Address information for the report
b) Directory paths where ToxRat will search for workbooks and masterbooks
c) Directory path for the settings files (= settings for statistical methods for specific workbooks)

The file **Tables.dll** contains statistics tables, **all other dll files** contain biotest-specific and method-specific texts. The dll files are accessed during the evaluation process, which requires read rights for the program directory.

The registration dialogue will appear when the software is initialised for the first time. Enter the unique username and registration key you were given when you purchased your license.

ToxRat will then continue the installation. A welcome screen (Figure 104) is displayed when the software is initialised for the first time, and the following two user-specific directories will be created (which is why the program must be initialised using the account of the user, that ToxRat will later be using);

C:/User/<username>/Documents/ToxRat Professional

C:/User/<username>/Appdata/Local/ToxRat Professional

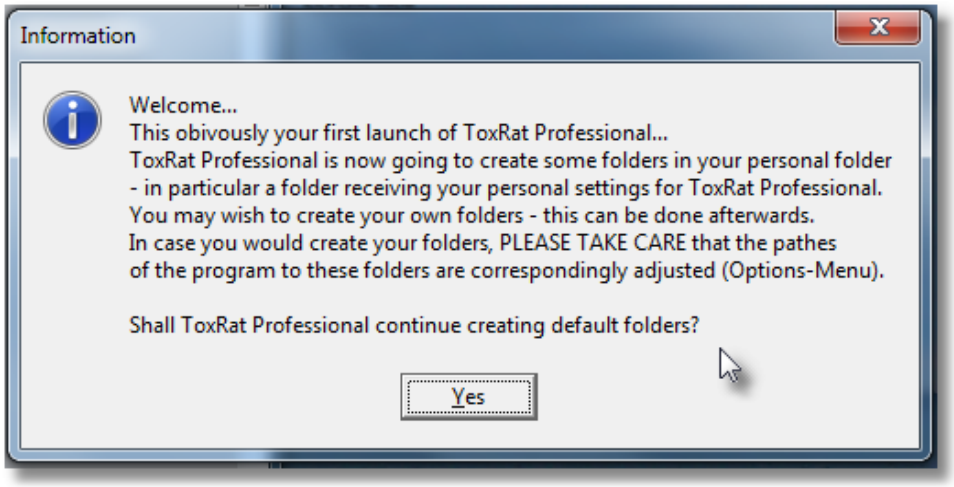


Figure 104: Welcome screen at initial program start.

Re.: 1) **C:/User/<username>/Documents/ToxRat Professional** (Figure 105)
ToxRat will suggest this directory as the storage location for reports the user will generate in future, and this is also where ToxRat will automatically search for user-specific workbooks (as opposed to the demo workbooks that can be found in the program directory). The folders "Report" and "Workbooks" will initially be empty. You can modify the location of the target folder at any time via "Options" >> "Input-Output", once the installation is complete. It might, for example, be a good idea to use the demo workbooks provided, while you are still finding your way around the software. Simply change the path for the workbooks back to the demo folder in the program directory (Figure 107). You could alternatively create copies of the demo folders in your user-specific directory.

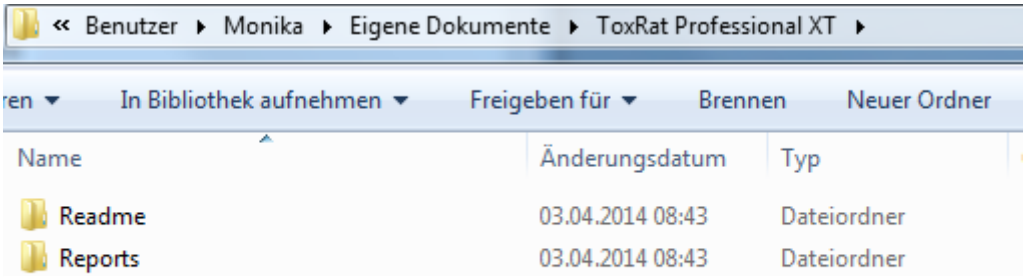


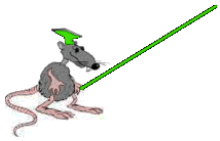
Figure 105: Folders created automatically under "My Documents" during installation; these will later be used as storage location for your own workbooks and reports.

Re.: 2) **C:/User/<username>/Appdata/Local/ToxRat Professional**

This directory will always contain the following:
Temporary graph files with the file extension .wmf. Any graph created during a software run will be temporarily stored here in wmf format. All wmf files are automatically deleted when ToxRat is closed.

The following user-specific program settings ("personal settings") can also be found in this directory. This path is optional and you can change it at any time via "Options" >> "Input-Output" (Figure 107):

The file **ToxRat.dll** contains the report address, the settings file path, paths for the demo workbooks and masterbooks provided, and paths for user-specific workbooks and reports (see above). The welcome screen "Initialisation" (see Figure 104) will be displayed if the file ToxRat.dll is not found in this directory, and it will then be copied to this location from the program directory (initially containing default paths and directories).
The **settings file ToxRatPro3.0.0.stp** contains system default settings for the various statistical methods and biotests. The file ToxRatPro3.0.0.stp will be created if it is not found at program initialisation.
Biotest-specific settings files with the file extension .stp. In this directory, ToxRat creates an stp file for every workbook type as it is called up for the first time. The file contains system default settings for the evaluation. Any changes effected by the user will be permanently saved in the workbook-specific settings file, and will be automatically applied when a workbook of the same type is called up the next time.
The stp files should be saved in user-specific directories to ensure that several users can always find and apply their own individual settings. When a workbook type is called, for which no stp file exists, then ToxRat will create one for you (with the system default settings)



Note for users of previous ToxRat versions: As of ToxRat 3.0, user-specific settings for statistics methods and biotests will no longer be stored in one central settings file, and will be stored for specific biotests instead. That means that only those settings files will have to be renewed in future updates that have had changes applied. The settings for all other biotests can be applied to the update directly via the specific settings files (for example complex report profiles).

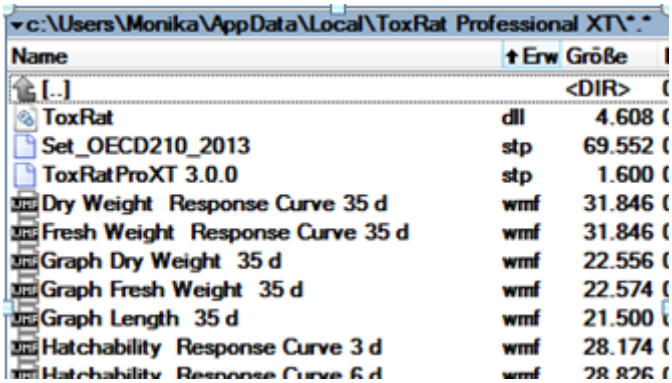


Figure 106: User-specific folder for storing settings files (*.dll, *.stp, permanent) and graphics files (*.wmf, temporary).

The program will start as soon as these two directories have been created. It will not require administrator rights for any subsequent program calls.

You will now see an overview of all created directories and the system default paths (Figure 107) under the menu item "Options >> Input-Output". You will be able to make some changes, but you will need administrator rights if you want to change some of the main paths.

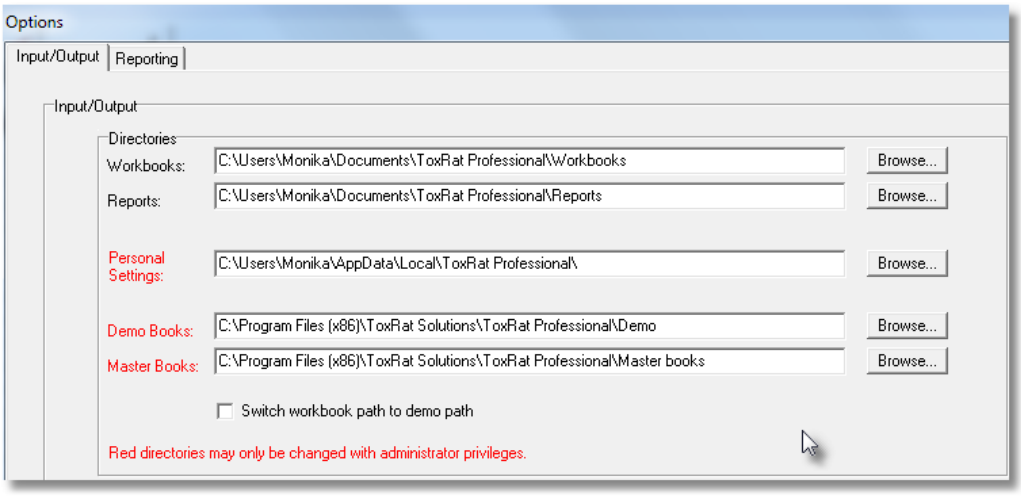


Figure 107: Setting options for directories and folders under the menu item "Options >> Input-Output"; here: system default settings.

9.2 Server Installation

ToxRat will run on a client-server network (all computers in the network must run Windows OS) or a terminal-server network (computers in the network have no OS installed).

Make sure to read the information provided for local installations first - the directories created and the procedures used apply for the server installation as well. Run the ToxRat installer on the server.
In a client-server configuration, you should then run the program Client.exe on each individual computer in the network. The client-program copies specific system files required for running the program to the Windows system directory on the local PC. Call up ToxRat on each computer in the network using the username of the relevant user - you will need administrator rights when ToxRat is initiated for the first time. The user-specific file structure described in chapter 9.1 will then be created on the local computer. Some of the paths can be modified later - for the settings files, the demo workbooks and masterbooks this will require administrator rights.

Please note the following for a server installation:

1. Make sure that the users have read rights for the dll files in the program directory on the server.
2. The demo and masterbook files included in the scope of delivery will be stored in the program directory on the server by default. If you prefer having these files available in another central location, simply copy the relevant directories to the new location; don't forget to set the new paths in the "Options" menu on each local computer in the network.
3. It is advisable for workbook and biotest-specific settings files to be saved individually by each user to ensure that one user doesn't overwrite the settings of another user. Each user will need read/write rights for the relevant directory.

Uninstalling ToxRat

A deinstallation will only remove the relevant program directory and the files contained in the system directory. The folders in the user-specific directories (see chapter 9.1) will remain unchanged.

Biotest Workbooks included in ToxRat

Please find below a list of all biotests for which templates (workbooks and masterbooks) are available in ToxRat 3.0. (Please remeber: the scope of templates included in the delivery depend on the ToxRat variant, see chapter 2).
For biotests which may be performed in several variants (e.g. OECD 201: cellcounts, extinctions, fluorescence; OECD 211: flow through, semistatic), also several templates are available, to be identified by the file name. The complete list will be availbale when using the file menu.

OECD 201	Algae, Growth Inhibition Test
OECD 202	Daphnia spec., Acute Immobilisation Test
OECD 203	Fish, Acute Toxicity Test
OECD 204	Fish, Prolonged Toxicity Test: 14-Day Study
OECD 205	Avian Diatary Toxicity Test
OECD 206	Avian Reproduction Test
OECD 207	Earthworm, Acute Toxicity Test
OECD 208	Terrestrial (Non-target)-Plant test:
	208A: Seedling Emergence and Seedling Growth, 208B: Vegetative Vigour Test
OECD 209	Activated Sludge, Respiration Inhibition Test
OECD 210	Fish, Early-Life Stage Toxicity Test
OECD 211	Daphnia magna Reproduction Test
OECD 212	Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages
OECD 213	Honeybees Acute Oral Toxicity Test
OECD 214	Honeybees Acute Contact Toxicity Test
OECD 215	Fish, Juvenile Growth Test
OECD 216	Soil Microorganisms, Nitrogen Transfomation Test
OECD 217	Soil Microorganisms, Carbon Transformation Test
OECD 218	Sediment Water Chironomid Toxicity Test Using Spiked Sediment
OECD 219	Sediment Water Chironomid Toxicity Test Using Spiked Water
OECD 220	Enchytraeidae Reproduction Test
OECD 221	Lemna sp. Growth Inhibition Test
OECD 222	Earthworm Reproduction Test (Eisenia fetida / andrei)
OECD 223	Avian Acute Oral Toxicity Test
OECD 225	Lumbriculus Toxicity Test
OECD 226	Predatory Mite (Hypoaspis) Reproduction Test in Soil
OECD 227	Terrestrial Plant Test Vegetative Vigour

OECD 235	Chironomus Acute Test
OECD 236	Fish Embryo Acute Toxicity Test (FET)
OECD 237	Honeybees Larval Toxicity Test
OECD 238	Myriophyllum spicatum Sediment-Free Toxicity Test
OECD 239	Myriophyllum Water-Sediment Toxicity Test
OCSPP	850.5400 Algal Toxicity - Tiers I and II
IOBC	Mortality and Reproduction of Typhlodromus pyri
IOBC	Survival and Viability of Aphidius rhopalosophi
DIN EN ISO 11348	Luminescence Inhibition
DIN EN ISO 15088	Acute Toxicity of Waste Water to Eggs of Danio rerio
DIN EN ISO 17512-1	Earthworm Avoidance Test
DIN EN ISO 17512-2	Collembola Avoidance Test
DIN EN ISO 20079	Duckweed Growth Inhibition
DIN EN ISO 6341	Daphnia Acute Test
DIN EN ISO 7346	Fish Acute Toxicity Test
DIN EN ISO 8692	Algae Growth Inhibition Test
DIN 38412 L9 + L33	Algae Growth Inhibition
DIN 38412-L30 + L11 + L40	Daphnia Acute
DIN 38412 L31 + L15	Fish Acute
DIN 38415-T6	Fish Eggs
DIN EN ISO 06341	Daphnia Acute Test
DIN EN ISO 8692	Algae Growth Inhibition
DIN EN ISO 11348	Luminiscent Bacteria Test
ISO WD 11350	Ames Fluctuationtest
ISO 10706	Daphnia magna Longterm Test
ISO 10871	Solid Contact Test with Arthrobacter globiformis
ISO 10872	Caenothabditis elegans
ISO 11267	Inhibition of reproduction of Collembola
ISO 11350	Ames Fluctuation Test
ISO 16191	Myriophyllum aquaticum Sediment Test
ISO 20665	Ceriodaphnia Population Growth Inhibition Test

Additionally, ToxRat provides unspecific workbooks for metric and quantal data and for evaluation oft he Parallel Line Assay (Potency Estimation) according to Pharmacopeia.

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