New Statistical Procedures

Hampel Outlier Test

ECx calculation of metric data by way of non-linear regression
- 2-3-4 Parameter Normal, Sigmoid (Bruce Versteeg)
- 2-3-4 Parameter Logistic
- 2-3-4 Parameter Weibull
- Weightings: relative, Poisson, by variability
- Optimisation methods: Levenberg-Marquardt, Downhill-Simplex
- Confidence limit calculations: Monte Carlo Simulation, Bootstrap procedure

ECx calculation of quantal data:
- Spearman-Kärber / Trimmed Spearmann Kärber

Multiple tests for NOEC calculation:
- Stepdown Jonkheere-Terpstra
- Bonferroni Median Test
- Stepdown Cochran Armitage

Tests for monotony
linear+quadratic contrasts
(Rao-Scott-) Cochran Armitage Trend Test
Jonckheere-terpstra Trend Test

Tarone test for **extrabinomial variance**

New and updated Workbooks and Masterbooks
A complete list of all available biotest data templates can be found in the manual.

New Biotest Workbooks
OECD 205 Avian Diatary Toxicity Test
OECD 206 Avian Reproduction Test
OECD 213 Honeybees Acute Oral Toxicity Test
OECD 214 Honeybees Acute Contact Toxicity Test
OECD 223 Avian Acute Oral Toxicity Test
OECD 235 Chironomus Acute Test
OECD 236 Fish Embryo Acute Toxicity Test (FET)
OECD 237 Honeybees Larval Toxicity Test
OECD 238 Myriophyllum spictum Sediment-Free Toxicity Test
OECD 239 Myriophyllum Water-Sediment Toxicity Test

IOBC Typhlodromus
IOBC Aphidius

ISO 10872 Caenorhabditis
ISO 10706 Daphnia magna Reproduction
ISO 20665 Ceriodaphnia
ISO 16191 Myriophyllum aquaticum

DIN EN ISO 15088 Eggs of Danio rerio
DIN EN ISO 17512-1 Earthworm Avoidance
DIN EN ISO 17512-2 Collembola Avoidance

OPPTS (=OCSPP) 850.5400 Alga Toxicity

Updated biotest workbooks (Adaptations to more recent guidelines)
OECD 209 Activated Sludge Respiration Inhibition Test (2010)
OECD 210 Fish Early Life Stage Test (2013)
OECD 211 Daphnia magna Reproduction Test (2012)

Changes in Specific Biotests

Algae Test Workbooks based on Flourescence (OECD 201, DIN 8692)
The calibration sheet for flourescence has changed. The slope now is obtained by linear regression through the origin. Threfore, workbooks using previous calibration sheets (ToxRat 2.10.05) cannot be processed anymore using ToxRat 3.0, unless the calibration sheet is replaced by the former one. Moreover, depending on calibration data, the results obtained with ToxRat 2.10.05 and ToxRat 3.0 might be different.

OECD 201
Additional workbooks for specific test or metering designs:
"OECD201 AlgaeGrowthInhibition(Extinction duo).xls"   Allows the input of different calibration curves for two different ranges of extinctions.

OECD 209
ToxRat contains two separate OECD 209 workbooks - one for parallel, and one for serial measurements. For the latter, the UBA (Federal Environment Agency) document[1] on the new OECD 209 guideline recommends a two-sample test between the controls measured at the start and at the end of the measuring series. This will verify, whether the background respiration rate has changed during the measuring time. Any changes will be documented, but further evaluations will be based on all six control replicates if the user decides to use "pooling".

OECD 210
Now contains a data input sheet for both dry weight and fresh weight. Fresh weight and dry weight can therefore be evaluated simultaneously; alternatively, one (or both) sheet(s) can be left empty.

OECD 211
In accordance with the updated test guideline of 2012, a trend test will check for significant mortality. ToxRat 3.0. automatically calculates both the cumulative offspring of introduced (COI) and the cumulative offspring of survivors (COS). The user will have to decide which end point should be reported depending on results (according to the guideline: COI in case of significant mortality, COS in case of non-sigificant mortality).

ToxRat 3.0. is able to process unbalanced designs, i.e. any replicate number in control and treatments.

---

[1] "Frage und Antwort Dokument des Umweltbundesamtes zur neuen Richtlinie OECD 209: "Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation) (22.7.2010), Version vom 2.3.2012"

OECD 218/219

New input routine for the verification of the number of larvae introduced, as their number cannot be smaller than that of larvae emerged. A relevant correction was already introduced in ToxRat 2.10.05, but the associated data consolidation is now more transparent: ToxRat will create additional data sheets and stores the modified file with a specific suffix. Please see the manual (chapter 5.3, p 80) for details and consequences for handling of the corresponding workbook, specifically when inputting new data.

Compatibility with Predecessor Versions

All existing workbooks and masterbooks have been revised; the information in the worksheet General Notes, which control the evaluation, has been standardised. ToxRat 3.0. is generally downward compatible, i.e. you should be able to read and evaluate workbooks from predecessor versions without any problem. We do, however, strongly suggest to use only the new templates for new data to be added, as all of the functions have only be tested for these new templates. You will see a warning message when reading a workbook from ToxRat 2.10.05 or lower in ToxRat 3.0., and you will be asked to use one of the new templates instead. The workbooks and masterbooks that were developed or updated specifically for ToxRat 3.0 (see list above), cannot be read or evaluated by previous ToxRat versions! ToxRat 3.0 still requires the file format xls or xlt. If you use MS-Excel to fill in or edit ToxRat workbooks or Masterbooks, please ensure to save as xls or xlt, not xlsx or xltx.

Manual

The manual was rewritten in its totality, and is now available in both German and English. The structure of the manual follows the practical procedure used in a biotest evaluation. It not only describes the programme functionalities, but also explains test selection criteria and the meaning of the achieved results.

Validation

Since several statistical methods were added or edited, ToxRat 3.0 needs to be again validated. Sample-results from all implemented statistical methods are listed in chapter 6 of the validation document („Results for Routine Validation of ToxRat Computation") and can be reproduced using the files validation data 1-6, which are included in the terms of delivery.

In addition to the routine validation, in chapter 1-5 of the validation document all implemented statistical methods are described, their algorithms are listed and the results are compared to results from literature or to independent calculations. With non linear regression, currently the Levenberg-Marquardt-Algorithm is shown. The corresponding description of the Downhill-Simplex-Algorithm will be added later. Nevertheless, sample results for routine validation (see above) are included for both Algorithms.

Installation

ToxRat 3.0. can be installed with WIN XP or WIN7 in parallel to a previously installed ToxRat 2.10.05 version

Fundamental Changes and Additions in Comparison with the ToxRat Version 2.10.05

Automatic Evaluation (RUN) for Generic Response Workbooks

From version ToxRat 3.0 and higher, you will be able to initiate a software-controlled evaluation sequence with the push of a RUN-button via the menus "Find NOEC" and Find Limit Level" not only in the biotest workbooks, but also in the generic workbooks.

Menu Navigation

In generic workbooks, you will find the selection dialogue for determination of Effect levels (ELx) directly in 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 evaluation.

Significance Levels

The significance levels for pretesting and final testing can now be configured independently for a testing sequence to determine NOEC.

The default value for significance levels in pretests for normal distribution and variance homogeneity has been changed to 0.01 (previously: 0.05) in order to a) avoid an unnecessarily weak final test, and b) to avoid alpha cumulation.

Settings for Evaluations

ToxRat 3.0. will optionally generate a so-called settings table with every evaluation. This table documents the settings used for the current evaluation and shows, whether these settings differ from the system presets. The settings table will be displayed as the last results table (after "Summary").

The (predefined) settings to be used by ToxRat for an evaluation are stored in so-called "settings" files. These are now organised biotest-specifically, i.e. there is no longer one overall settings file for the entire system. Instead, individual settings files are created for each workbook type (e.g. set_OECD210.stp). ToxRat will read the settings from the associated settings file, whenever you open a specific workbook type. For the initial default settings, these will be the system presets, and for later uses these can then be your individually configured settings. That means that in future updates, you will be able to keep your individual settings files from previous versions for biotests without any changes.

Please note: Due to the complete reorganization of the biotest specific settings, ToxRat 3.0 **fails to save user specific report profiles**.

Non-linear Regression

ToxRat 3.0. includes methods for non-linear regression for metric data only, because for metric data in particular, non-linear regression is an important alternative for linear regression, as it can operate without normalisation (exception; 2-parameter CDFs). The problem of normalisation does not exist for quantal data, which is why linear regression will always be a good choice.

Since non-linear regression is recommended in a lot of guidelines, the ToxRat default settings for effect level determination for metric variables usually is 3-parameter-non-linear regression. However, In light of the high number of possible variants and settings options for non-linear regression, default settings may not suffice for a successful result, depending on dataset. You may in this case have to try several variants to come to a meaningful result. The manual contains an orientation regarding the factors that can be interchanged, as well as for the goodness of fit criteria for the assessment of the results. Please also note the sample results from the validation document (chapter 6.5): it gives you an idea of how the selection of function and algorithm affect the result using one and the same file each time.

Important: The Downhill-Simplex algorithm and its associated Bootstrap procedure for calculating the confidence limit is highly processing-intensive. Depending on dataset (number of variables and measuring times), it may take several minutes (!) to complete and for results to be displayed. Do not interrupt the programme as long as the activity icon is displayed! Tip: if you just want to check, whether the Downhill-Simplex Algorithm is generally suitable, you can significantly reduce the number of resamplings.

Programme Libraries

A number of biotest-specific text outputs have been stored in dll format in the programme directory in order to decrease the size of the ToxRat programme file. These dll files are accessed during evaluation runs, and that will require read rights (as before) in the programme directory.

Data and Result Tables

The data tables generated by the system during an evaluation can be accessed directly via the menu item "Tables" (as before). For reasons of clarity, however, the data tables will no longer be added to the workbook in the form of additional datasheets. That now applies only to the results tables ("Tabs<name of variable>").

Optional Additional Variable for "Quantal Responses": #escaped

The generic workbooks for quantal responses now allow an optional input of not only #dead, #survived# and #emerged, but also for #escaped. A relevant file template can be found in the demo workbook directory and in the masterbook directory.

Modifications of Existing Statistical Methods

Levene Test

In ToxRat 2.10.05, the Levene Test used quadrated residues for calculation $(x - x\_)^2$. That is one of several variants mentioned in the original publication[2] . SPSS and R utilise a different variant, namely the maximum absolute deviation $|x - x\_|$. ToxRat 3.0. also uses maximum absolute deviations in order to produce the same results as SPSS and R. As a consequence, you will find that the results of the Levene Test will differ between ToxRat 2.10.05 and ToxRat 3.0.

Confidence Limit Calculations

In linear regression for metric data, there is now an option to include the control variance in confidence limit calculations (in accordance with Annex 5 OECD 201). The confidence ranges will therefore generally become somewhat broader. This variance correction is activated by default - it can, however, be optionally deactivated in order for you to reproduce the results from predecessor versions of ToxRat (see manual for more information).

Bootstrapping is now also available as an additional procedure for the confidence limit calculation with linear regression for metric data.

A function will now allow the calculation of the t value to up to 14 decimal spaces for the calculation of the 95% confidence limit in linear regression. In earlier versions, the t value from the normal distribution table was used (rounded to two decimal spaces = 1.96). The increased accuracy of the value may result in a slight deviation of the confidence limit values calculated with ToxRat 3.0. in comparison with calculations done in previous versions.

Linear Regression, Algorithm: Maximum Likelihood Regression – number of RUNS, weights

To increase the precision of regression even if there is no clear dose response, the stop criteria for the iteration process was modified. Depending on the data set, this may result in a different

---

[2] Levene, H. (1960): Robust tests for equality of variances. In: Contributions to Probability and Statistics. Essays in Honours of H. Hotelling, Stanford, 278-292

number of RUNs and thus different weights (generally, the weights of the last RUN are shown). As a consequence, also the values for F and thus p(F) may be altered compared to ToxRat 2.10.05.

Lineare Regression, Algorithm: linear weighted regression – weights
The function for determination of weights has been replaced by a function with increased accuracy. Depending on the data set, this may result in different weights and thus different values for F and p(F) compared to ToxRat 2.10.05.

Linear Regression, calculation of p(F)
The function for determination of p(F) has been replaced by a function with increased accuracy. Therefore, even if the F values of ToxRat 2.10.05 and ToxRat 3.0 agree, the values for p(F) might be slightly different in ToxRat 3.0.

Minimum Detectable Difference (MDD) Calculation
The function for calculating the t-quantiles for the used significance level (t(crit) values) has been replaced. For degrees of freedom up to 4, the new function is more accurate than the function used in previous ToxRat versions. The resulting MDDs from ToxRat 3.0. will be smaller than those from previous ToxRat versions. Important: This applies for t(crit) values for calculating MDDs only, and not for the calculation of sample-t-values, which are used for the testing of significance. The sample-t-values are the same as in previous ToxRat versions.
The function to determine the p(t)-values has been replaced also. The new function allows to calculated exact p(t)-values below 0,001, i.e. for very high t-values, for which previously only "zero" was returned.
As a consequence, for data sets showing very high t-values, in two sample tests the resulting MDDs from ToxRat 3.0. may slightly differ from that of previous ToxRat versions. In multiple tests with Bonferroni correction, the higher precision of p(t) values smaller than 0,001 may affect the sequence for calculation of the corrected significance level alpha(i). This may also result in slightly different MDDs compared to previous ToxRat versions.
Please note: This may only affect the MDDs – the results of the significance tests are the same as before.

Williams Test, Calculation of Likelihood Means (LhM)
The likelihood-means are calculated without consideration of the control (as in 2.10.05), but now the calculation depends on the selected test direction.
For usual data sets and testing scenarios (decreasing means with increasing concentration, test direction one sided smaller / or increasing means with increasing concentration, test direction one sided greater) this will not affect the results.

NOEC Determination
A heterogeneous significance situation will result in the output "expert judgement required"; the system will not suggest a NOEC value.

F Test Comparisons for Two-Sample and Multiple t Tests
Previously, the F test was conducted one-sided. ToxRat 3.0 uses a two-sided test for the comparison of two variances to avoid an unnecessarily rejection of variance homogeneity in F tests.

Bug fixes

A number of minor function errors have been corrected, which we will not all list individually. We will only mention those bug fixes that have had an influence on the results:

OECD 201
Previously, when the "Yield" value was exactly "-1", the result was interpreted as a "missing value" instead as value → that was corrected.

OECD 208
In specific data constellations, the validity criteria "Seedlings Emergence" and "Seedlings Survival" were incorrectly stated as 0%. That was corrected.

OECD 218/219
In ToxRat 2.10.05, the value "-0.5 days" was used incorrectly as the start age for the introduced larvae. The correct value is "0.5 days". The negative value would result in an overestimation of development rates. Since, however, any unified start age set is arbitrary, and since the day logged as emergence day similarly can only be accurate to between +/-24 hours, the developmental rates anyway are only approximations.

In ToxRat 2.10.05, every calculation was based on the same starting age of the introduced larvae (-0.5), regardless of what was stated in the window "Options / OECD 218/219". The start age of introduced larvae can now be edited by the user, which means the value in the "Options" window will actually be used. The default value here is 0.5 days.

Linear Regression, p(F)
If F < 1, ToxRat 2.10.05 didn´t present the p(F)-value, but the result of „1 minus p(F)". E.g. if F=0,9, ToxRat didn´t present the (correct) p(F) = 0,42 (42%), but 1-0,42 = 0,58 (58%). This has been fixed.
F-values smaller than 1 only occur if there is no dose response relation at all. With F <1, regardless of the test design (degrees of freedom), both values for p(F) and 1-p(F) generally clearly exceed the significance level of 5% (see example above). Therefore the bug did not affect any of the conclusions from linear regression of ToxRat 2.10.05.