Useful methods

Phytotoxicity is one of the fields of duckweed research currently represented by far more scientific publications than any others. One can screen this easily by reading through the section “From the database” in the several issues of our newsletter “Duckweed Forum”. Meanwhile, it is not any more satisfying just to report inhibitory effects of heavy metals, herbicides or other xenobiotics on growth of duckweeds evaluated by frond number, fresh weight or dry weight. As a minimal requirement, these inhibitory effects should be quantified. From effect curves (e.g. number of fronds over the time of treatment depending on the concentrations) and calculating the different exponential growth rates, dose-response-curves or dose-inhibition-curves can be created, commonly as log dose versus inhibitory effects. However, this must not be the end of the evaluation but just the beginning. With suitable statistical methods, effective concentrations (EC) can be calculated together with suitable form of errors. Depending on the requirement of the field, EC values should be calculated at different effect levels like 50 or 10 percent of inhibition. Only such quantitative data offer the opportunity to compare the toxic effects of different herbicides or other substances and also of physical effects like UV or radioactive irradiations. Also, when the sensitivity of different species or strains of duckweeds should be compared, such quantitative evaluation is indispensable. One programme, called ToxRat®, which can be used for such evaluations (and other applications) is explained in the following by Monika Ratte. Additionally to the references given there, we recommend the following papers that show applications in the field of duckweeds:


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ToxRat: Custom-designed Software for Statistical Evaluation of Duckweed Growth Inhibition Test

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Duckweed species are used in ecotoxicology to determine the toxic effect of chemicals or waste water on aquatic plants. During the course of usually seven days, frond numbers, frond areas or biomass are determined in different test concentrations and in a control. Growth rates are calculated and the growth rates obtained in treatments are compared to those measured in the control.
Biology meets statistics

Subsequently, the data needs to be statistically evaluated. Specific effect concentrations causing x% inhibition are calculated by regression methods and/or so called No Observed Effect Concentrations (NOEC) are determined by statistical testing.

Numerous statistical packages are available to perform these evaluations. However, these usually are designed for a comprehensive application range, rather than exclusively for ecotoxicology, not to say especially for the Duckweed growth inhibition test.

From biologists for biologists

This was the situation in 2002, when Hans Toni and Monika Ratte, two biologists from the Technical University of Aachen, felt the necessity of an interface between ecotoxicology and statistics. "Our vision was to enable both routine evaluation and correct statistical evaluation of biotests even for not-statisticians" says Monika Ratte, who takes care for the customer support. As a result, the software ToxRat was developed: a user-friendly software for the practitioner who wants to perform statistical analysis of biotests according to international guidelines (Figure 1).

The programming work has been and is still done by Hans Toni Ratte, associate Professor at the Institute for Environmental Research of the Technical University of Aachen until 2011. Here, he was involved in statistical education of students and attended a lot of boards and committees as expert for ecotoxicology and statistics.

What makes ToxRat different to other statistical program packages?

1. ToxRat "knows" the data structure and experimental design of individual biotests.

When starting the program, the user simply selects a biotest from a list of available templates. E.g., for Duckweed data, the user might select either the Lemma Growth Inhibition Test according to OECD 221 (2006) or the Duckweed Growth Inhibition Test according to DIN ISO 20079 (2005) (Figure 2).

Thereby a tailor made template for data input will be opened, predesigned to the data recorded in a Duckweed biotest (Figure 3). The template actually is an MS-Excel file, containing different sheets: "General notes" for basic information about the experiment, test item, unit of test item concentration etc. and several sheets for raw data, such as frond number, frond area and biomass. The treatments
are ordered in columns, starting with the control, the replicates are ordered in lines. All general terms such as variable name, number of replicates and even the measurement intervals have been pre-set in accordance with the corresponding guideline (but of course might be edited and changed, if desired). So, the user can easily enter the measurement results directly into the data template or transfer the data via copy-paste.

2. **ToxRat also “knows” biotest guidelines**, e.g., which toxic metrics are required by the guideline and which validity criteria are prescribed. As a result, the user doesn’t need to select any statistical method or to perform any mathematical calculation, but can make use of tailor made default settings for the Duckweed growth inhibition test: Pressing the RUN Button (Figure 4), starts a complete evaluation sequence, including validity check (Figure 5), calculation of yields and growth rates from the raw data, percentages of inhibition, regression for ECx calculation and multiple testing just as prescribed in the corresponding guideline. Thereby, up to six user defined EC levels can be selected (Figure 6). For these, also the 95% confidence limits are calculated (Figure 7). Finally, ToxRat generates a comprehensive report with graphics and tables as an rtf or pdf file.

3. **ToxRat includes expert knowledge about statistics.**

ToxRat takes care for selecting appropriate statistical methods. Thereby it focuses on methods used for statistical evaluation of single species biotests in ecotoxicology according to the OECD guidance document No. 54 (OECD 2006).
Individual biotest guidelines are updated from time to time and statistical methods might change over time. E.g., ECx calculation for growth rates obtained in a Duckweed growth inhibition test has been and is still performed frequently using linear regression, namely Probit analysis. However, in the mean time, more and more new and updated guidelines recommend to use non-linear regression rather than linear regression for metric data such as growth rates (e.g. OECD 201, 2011). This is due to several reasons, amongst others, normalizing of the treatment means by the mean control response is regarded to pose some principle problems (Green 2016). Even in the OECD guideline 221 (2006) for the Duckweed growth inhibition test, the non linear regression is mentioned as "preferred technique". The ToxRat software enables Probit-, Logit- and Weibull-analysis using linear regression, but following the recent state of the art, the default settings are preset to non linear regression using either 2-,3- or 4 parameter functions (Figure 8). Thereby, the default settings should be regarded as recommendations, which can be changed by the user, if desired.

![Graphical output of a linear (left) and a non linear (right) regression with growth rate data of variable frond number](image)

**Figure 8:** Graphical output of a linear (left) and a non linear (right) regression with growth rate data of variable frond number

The OECD test guideline No. 221 for the Duckweed growth inhibition test prescribes also the threshold concentrations, called NOEC, to be calculated as a toxic metric. However, no certain recommendation is given on which statistical tests should be used, except referring to the OECD document No. 54 (2006) for guidance. Also here, the user can rely on ToxRat: the software offers an automatic mode, running a complete sequence of pretests and selecting an appropriate final test depending on the results of the pretests. So, using default settings for evaluation in any case ends up with correct results. This enables evaluation of data sets even for not-statisticians and saves a lot of time with routine evaluation. Moreover, ToxRat is even recommended in an OECD guideline as appropriate software for statistical evaluation (OECD 243).

Though default settings will produce statistically correct results in either case, it goes without saying, that depending on the data, user settings might be required in order to further optimize evaluation. For this purpose, the advanced user can select individual settings, both for ECx calculation and NOEC determination (Figure 9).
4. Service and Support available

ToxRat is not simply just a software program. According to the principle that "a software is just as valuable as the support behind", the ToxRat team places the highest value on prompt and qualified customer service and effective, individual advice and assistance with software operation, data analysis, selecting an appropriate statistical method and interpreting the results.

![Image of software interface](image.png)

Figure 9: NOEC determination: Default settings available – user settings possible

5. Validation

The scope of delivery includes a validation document explaining all methods and mathematical formulae used by the program. All mathematical and statistical procedures are applied to standardized test data sets, and the results are verified in comparison to the results of the independent calculations (e.g. MS Excel) and data published in literature. The test data sets form part of the scope of delivery of the software, which means that the users can repeat this quality control step to verify that the software delivers the correct results as stated in the validation document, also in the user’s individual system configuration.
Get your own impression

Trial versions are available at www.toxrat.com. They are free-of-charge and without any obligation. They enable to enter own data, to use all the settings, to carry out complete evaluations and to generate reports.

References


OECD 221 (2006) OECD Guidelines for the testing of chemicals. Lemna sp. Growth Inhibition Test

OECD 201 (2011) OECD Guidelines for the testing of chemicals. Freshwater Alga and Cyanobacteria, Growth Inhibition Test

OECD 243 (2016) OECD Guidelines for the testing of chemicals. Lymnaea stagnalis Reproduction Test