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Fish Acute Toxicity Test: The number of animals can be reduced

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In the fish acute toxicity test (OECD Guideline 203), animals experience a high level of pain when exposed to lethal concentrations of a test compound. Any measures for reducing the number of animals in the test without loss of information should be strived for. With this goal in mind and based on long standing observations, Dr. Hans Rufli from* **ecotox***solutions, Basle, Switzerland, initiated the present project supported by the 3R Research Foundation (No. 114-08).*

After the analysis of historical data from two databases and a simulation procedure, an international expert group concluded that fish acute toxicity tests can be performed with fewer animals than indicated in the present OECD guideline.

Hans Rufli

Timothy Springer

Hans Rufli
rufli@ecotoxsolutions.com

ecotox*solutions*
Unterer Rheinweg 114
CH-4057 Basel
Switzerland
http://www.ecotoxsolutions.com

Analysis of historical data

It is not uncommon in routinely performed fish acute toxicity tests to find very steep dose-effect relationships in which only one concentration causes a partial mortality (mortalities >0% and <100%) or even none (Fig. 2c). Accordingly, little information is gained from the 4 other concentrations with no or complete mortality. Therefore, the question arose whether this observation can be substantiated by an analysis of historical data derived from a laboratory database from industry (chemicals, pesticides, dyes, plastics, pharmaceuticals etc.), and the database of the Office of Pesticide Programmes (OPP). Dr. T. Springer performed the analysis of the data and test simulations, assisted by the statisticians Drs. J. Green and P. Chapman, previously involved in the Avian Acute Toxicity Test, which evaluated new designs with fewer birds. The results were then discussed in a workshop, together with alternative ways of reducing fish in case range-finding studies are required. Attendees as listed in Table 1 represented experienced experts from academia, industry and governmental authorities.

Slope and spacing factor in fish tests

The approximate LC50 and slope of the test compound concentration-response curve is assessed in an initial concentration range-finding study. Subsequently, a definitive test is performed either as limit test (no toxicity in range-finding: LC50 >100 mg/L or >water solubility limit) or full test (toxicity in range-finder: LC50 = x mg/L). The estimated inclination of the slope derived from the concentration range study determines the spacing factor between concentrations in the full test. At least two partial mortalities are necessary to estimate the LC50 using Probit analysis and to obtain an estimate of the slope. As an example, a steep slope of 13 (Fig. 2a) and a spacing factor of 1.6 (OECD recommends ≤2.2) or a flat slope of 6.5 (Fig. 2b) with a spacing factor of 2.1 both would yield two partial mortalities. Increasing the factor beyond 1.6 with a steep slope of 13 (or beyond 2.1 for a flat slope of 6.5) would yield only one partial mortality, and eventually none (Fig. 2c). Thus, increasing the spacing factor too much, most of the concentrations are wasted as they result in either 0 or 100% mortality.

Figure 1: Fish testing facility

It was observed that in practice too high spacing factors are often used covering a too wide concentration range, this in order to get a value for LC0 (highest concentration with no mortality) as well as for LC100 (lowest concentration with 100% mortality). By doing so, animals are wasted because up to 5 concentrations with 7 fish each result in 0 and 100% mortalities and preclude a regression analysis for an estimation of the LC50.

Historical data from industry

The observation of generally steep slopes was confirmed for data derived from the industry laboratory (median slope of 13, comparable to Figs. 2a and c). In 75% out of 329 studies, carried out according to standardized OECD guidelines, no or one partial mortality was reported (Fig. 2c). This means that Probit analysis of the LC50 was possible only in 25% of the studies. Furthermore, in 75% of the studies, at least two concentrations with 0 or 100% could have been omitted as they do not contribute to the calculation of the LC50. Clearly, spacing factors chosen are often too high. Consequently, fish are wasted.

Table 1. Expert panel at the workshop to reduce the number of fish in the acute toxicity test.

Dr. Böttcher M	IBACON	Germany
Prof.Dr.Braunbeck	Univ. of Heidelberg	Germany
Dr. Chapman P	Formerly Unilever	England
Dr. Green J	Dupont	USA
Jeram S	ECVAM	Italy
Dr. Länge R	BayerHealthCare	Germany
Dr. Maak G	UBA	Germany
Dr. Memmert U	Harlan Laboratories	Switzerland
Dr. Navas J	INIA	Spain
Dr. Polleichtner C	UBA	Germany
Dr. Rufli H	ecotoxsolutions	Switzerland
Dr. Schäfers C	Fraunhofer Institute	Germany
Prof. Dr. Segner H	University of Bern	Switzerland
Dr. Springer T	Wildlife International	USA
Dr. Straub J	Hoffmann-La Roche	Switzerland
Dr. Wheeler J	Syngenta	England
Dr. Zok S	BASF	Germany

Not confirmed by the OPP database

The OPP database with 4010 acute fish studies of pesticides (active ingredients, metabolites and multi-ingredient formulations) and 469 reported slopes of "core" studies did not confirm the steep slopes as found in the industry laboratory data. The median slope of the dose-effect relationship of 6.5 represents a relatively flat slope (comparable to Fig. 2b). The reasons for the different slopes of the two databases (median of 13 versus 6.5) are not known, but might be explained by different type of chemicals representing different modes of action (e.g. receptor mediated).

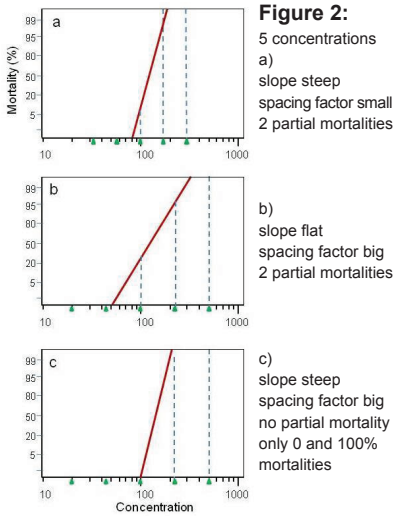

Figure 2:
5 concentrations
a)
slope steep
spacing factor small
2 partial mortalities

b)
slope flat
spacing factor big
2 partial mortalities

c)
slope steep
spacing factor big
no partial mortality
only 0 and 100% mortalities

Optimal study design with fewer fish derived from simulation study

Given the difference of the slopes of the two databases, it was not possible to propose a reduction of the number of concentrations. As a consequence, a Monte Carlo study (MC-simulation) was perfomed based on representative slopes and LC50-values of the historical data. Simulation offers a way to find an optimal experimental design without performing any additional *in-vivo* tests.

The current design of the OECD Guideline 203 (concentration range-finding: 4 fish, spacing factor 10; plus definitive test: 5 concentrations with 7 fish each, spacing factor 1.6) was compared with study designs of fewer concentrations (4), fewer fish in the definitive test (5, 6), and 2, 3, and 5 fish in the concentration range-finding study. The simulation showed that 6 fish per concentration yield the same quality of the LC50-value as a minimum of 7 fish presently required by the Guideline. Only in case of very flat slopes (e.g. <4), the quality may not be maintained.

Dose range finding: the most 3R relevant step

Further reductions in fish numbers were discussed at the expert meeting for the concentration range-finding process. This could be carried out by stepping-down from the UTC [1] reducing the number of fish by up to 70%. Furthermore, the use of fish embryos (48h FET) [2] instead of fish would lead to an additional reduction of 20%. Only the definitive test (limit or full test) has to be performed with fish (confirmation of fish embryo test data).

Implementation

The present proposals [3] could easily be put into practice immediately without a reduction of quality in the results. However, for global acceptance and implementation, an amendment of the OECD Guideline is mandatory. A lead country has to submit the proposed changes to the OECD with a Standard Project Submission Form (SPSF). At present, two SPSF have been submitted to the Federal Office for the Environment in Bern, Switzerland. One SPSF proposes a change of §17 of Guideline 203, Fish Acute Toxicity Test, from using "at least 7 fish at each concentration" to "6 fish", the other to replace the fish in the concentration range-finding process with fish embryoes. These changes will not only have a pronounced 3R-effect through an additional reduction of fish, furthermore, resources, time, cost and manpower will be reduced. For rapid global acceptance, the proposed protocol changes have to be explained to a wider audience in order to get support in meetings of the OECD.

References

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2. OECD Draft Proposal for a New Test Guideline on the 48h-Fish Embryo Toxicity Test (FET), May 30, 2006.
3. Rufli, H., Springer, T.A., Reduction of animal use in the OECD Fish Acute Toxicity Test, submitted to ET & C.

The findings elaborated with the present project were presented at the SETAC⁺-meeting in May 2010. For the proposal of using the fish embryo test instead of the fish juvenile test, the subject will be taken up during the OECD workshop on the Fish Testing Framework in September 2010.
⁺Society of Environmental Toxicology and Chemistry

* The acute fish toxicity test within the regulatory framework

Ecotoxicological tests are performed for industrial chemicals, agrochemicals (pesticides), biocides, and pharmaceuticals before they enter the market. For the registration and labelling of these substances, at the least they are subjected to an algae, daphnia and fish test for estimating adverse effects on the aquatic environment. The fish test is performed in a single concentration (limit test) at the lowest EC50-value of existing algae or daphnia toxicity data, set as threshold concentration (UTC) [1]. This is based on the fact that in about 80% of the substances studied, fish was the least sensitive group of organisms in the aquatic food chain. If mortality occurs in the limit test, a full fish study is required (5 concentrations). This should result in a dose-response relationship for mortality yielding an LC50-value for fish. However, for compounds specifically toxic to fish (e.g. certain agrochemicals), or if the test is started at 100 mg/L due to the lack of daphnia and algae data, a concentration range-finding study (pre-test) with a wide-spacing factor between concentrations of e.g. 10 is necessary prior to the definitive toxicity test.

REACH, the new EU Regulation on chemicals which entered into force on 1 June 2007, requires ecotoxicological testing for substances registered before 1981, which was not required before this date. The specific requirement for tests depends upon the properties and the quantity of substances manufactured per year. It is estimated that these tests require an additional 1.2 million fish per year. Therefore, it is an obligation to minimize the number of fish used in the acute fish toxicity test, in which mortality is the main parameter determined. The present project proposes ways to reach this goal.

REACH= Registration, Evaluation, Authorisation of Chemicals; EC= effective concentration; LC= lethal concentration.

3R Research Foundation Switzerland

Reduction, Refinement and Replacement
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Swiss Consensus Platform for 3R-Alternatives

www.forschung3r.ch

Office I Dorfplatz 5
P.O. Box 1372 I CH-3110 Münsingen
Phone +41 31 722 08 30
Fax +41 31 721 70 80
forschung3r@bluewin.ch

Scientific Information I Prof. Dr. P. Maier
P.O. Box 1372 I CH-3110 Münsingen
Fax +41 31 722 08 34
research.3r@bluewin.ch