4.8 Compilation of results of the ICPPR non-Apis working group with a special focus on the bumblebee acute oral and contact toxicity ring test 2014

ICPPR Non-Apis Working Group

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Abstract

Although honeybee risk assessment for chemicals has been rigorously revised recently, methods and techniques available for non-apis pollinators are scarce. An ICPPR working group "non-apis" was established in 2013 to address these knowledge gaps. Acute contact tests were designed and performed with solitary bees *Osmia* sp. but still require further optimization. Ring tests on acute oral and contact toxicity for the bumblebee *Bombus* sp. were developed and performed in 2014. Thirteen European laboratories participated in the trials and in most cases control mortality was < 10% after 96h, indicating that the developed methodologies were feasible in a variety of laboratories. The oral exposure and the group contact exposure tests were each found to generate more variable LD_{50} estimates, whereas the endpoints obtained in the single contact tests were more consistent among laboratories. The difference in the two different contact test designs indicates the presence of a 'housing' effect, which makes the group housing less favorable. In addition, the use of Tween80 as a wetting agent was found to be unsuccessful.

Introduction

The European Food Safety Authority (EFSA) has evaluated the current risk assessment of Plant Protection Products (PPP´s) on bees, resulting so far in an EFSA Scientific Opinion and an EFSA Draft Guidance document. Relevant gaps in the current tiered testing systems, especially concerning non-Apis bees, have been identified. Therefore, the development of reliable, scientifically sound and efficient testing methods for bumblebees and for solitary bees has gained a high level of importance. An ICPPR working group on non-apis bees was formed to address this problem at the SETAC Special Science Symposium "Plant protection products and pollinators: Testing methodologies, risk assessment and risk management" in Brussels, Belgium in October 2013. The final objective of the working group was to harmonize the test methods and make proposals for the upcoming OECD guidelines for testing chemicals on bumblebees, solitary bees and stingless bees. The work plan of the group included several phases, starting with the first tier acute laboratory tests and then continuing with the higher tier tests on bumblebees and solitary bees. Since the experience with stingless bees was extremely limited amongst the European participants, priority was given to bumblebees and solitary bees. The work on stingless bees was postponed and will be addressed later in cooperation with colleagues from, e.g., Latin America.

In March 2014, a workshop on the first tier test development for bumblebees and solitary bees was held in Niefern, Germany. As only fragmentary expertise in handling solitary bees in oral testing was available, this resulted in preliminary recommendations only and actual testing was postponed until 2015. It was decided, however, to start with a ring test for acute contact toxicity for solitary bees. As the results of this test were not yet consistent, further work on this subject was needed and is currently being planned. For the bumblebee testing, a working protocol for the acute oral and contact toxicity trials was distributed among the participating laboratories. The aim was to evaluate practical aspects and their impact on the results. In particular, the housing of the bumble bees, i.e. individual vs. group housing, was of interest since several participants had different experiences with the two methods. Presented herein are the results of these bumblebee trials.

Materials and Methods

General

Participating labs acquired the bumblebees (*Bombus terrestris*) from their own commercial suppliers. Hives were medium-sized, containing 60 to 80 workers. Test animals were constantly housed under test conditions (25 ± 2°C., RH 60 ± 10%; darkness). Maintenance and handling was done either under the red, day or artificial light. The animals were acclimatized for at least 12h and were tested within 1 week upon arrival in the lab. Before entering any test, the bumblebees used (or a representative portion thereof) were weighed. Very large and small bumblebees were excluded from testing. Since the bumblebee colonies are smaller than the honeybee colonies, multiple colonies were required for one test. As a consequence, bumblebees needed to be randomized over the treatments to avoid artefacts related to individual colony history, etc. Handling of the bumblebees occurred either without anaesthetics under the red light or with anaesthetics using CO_2 gas.

The test compound was dimethoate (EC 400) since this is the toxic standard in honeybee research. The dosage applied was checked by residue analysis of the dosing solutions. In all of the trials performed, only a 50% sucrose solution was provided for feeding. Pollen was not administered.

Acute oral toxicity trial

Test animals were acclimatized to test conditions overnight with access to a 50% sucrose solution (*ad libitum*). Before the feeding trial, bumblebees were starved for 2 to 4 hours. The test design included a control and five treatment groups, 0.25, 0.5, 1, 2 and 4 µg a.i./bee. The required amount of the test compound was spiked in a 40 µL 50% sucrose solution which was offered to the animals during the feeding period, immediately after starvation. The feeding period lasted for max. 4 hours and dosages were corrected for the actual food uptake. Per treatment group, 30 individually housed bumblebees were used. The observation period lasted for 96h and responses of the animals were recorded at 4h, 24h, 48h, 72 and 96h after dosing. During the 96h observation period, food (50% sucrose solution) was provided *ad libitum*.

The test was considered valid if <10% control mortality occurred after 96h.

Acute contact toxicity trial

In order to test whether group housing or individual housing of the bumblebees influenced the results, two separate contact toxicity trials were conducted. In both cases the test set-up included a control and five treatment groups, corresponding to 1.25, 2.5, 5, 10 and 20 µg a.i./bee. For each treatment group, 30 individuals were used, housed either individually (n=30) or in 3 groups of 10 bumblebees (n=3). Test animals were acclimatized to the test conditions overnight with unlimited access to a 50% sucrose solution.

Bumblebees were individually treated with a topical application of 5 µL of the appropriate dosing solution. Application was conducted using a micro-applicator, by placing the droplet on the dorsal side of the thorax of each bumblebee (between neck and wing base). As a wetting agent, most labs used Tween80 (0.5% v/v), and controls received the wetting agent as well. After treatment the animals were immediately transferred either to the group housing or individual housing systems.

The observation period lasted for 96h and responses of the animals were recorded at 4h, 24h, 48h, 72 and 96h after dosing. During the 96h observation period, food (50% sucrose solution) was provided *ad libitum*.

The test was considered valid if <10% control mortality occurred after 96h.

Data analysis

Although most of the mortality observed in the tests occurred in the first 48h, LD_{50} calculations were performed for the 96h time point, when the maximum effect had occurred. For the oral test, the LD_{50} was calculated, based on the actual mean dimethoate intake per treatment group. For the contact test, the LD_{50} was calculated as µg ai/bee. Calculations were performed using nonlinear regression models (2-or 3-paramater log-logistic models), in which mortality was expressed as proportion of dead individuals from the total number of bees in a group. For analysing the data from group contact tests, the repeated observations originating from the same housing cage were pooled.

All calculations presented herein were conducted in the R v3.1.0 statistical computing environment (R Core Team 2014). Dose-response models were fitted using the add-on package drc v2.3-96 for R (Ritz and Streibig 2005, 2013).

Results

Acute oral test

In total 13 oral tests were performed by 12 participating laboratories (fig. 1). Residue analysis confirmed that dosing solutions contained concentrations within 10% of the intended levels and consequently nominal concentrations were used in the analysis.

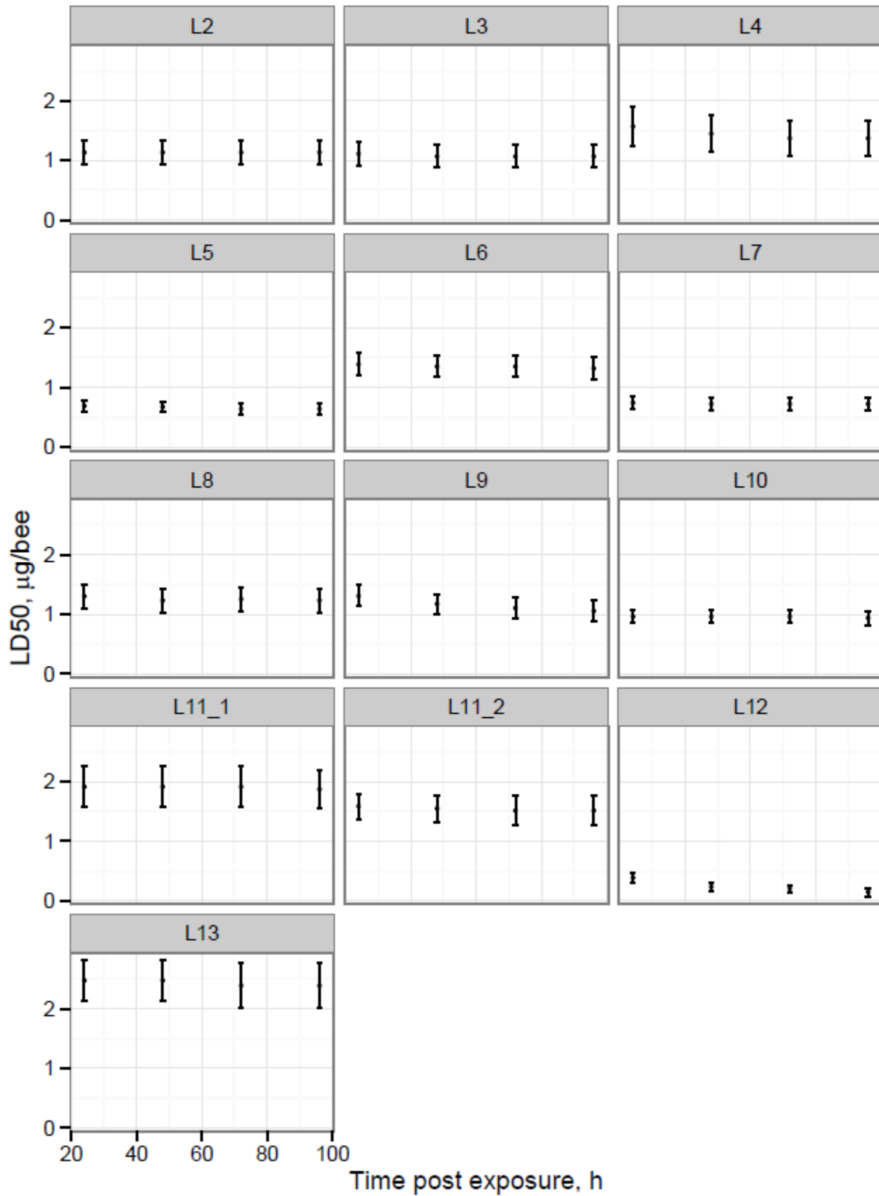

Figure 1 Point estimates of LD50 and their 95% confidence intervals obtained in the oral tests at 24, 48, 72 and 96 h after exposure in 12 laboratories (L).

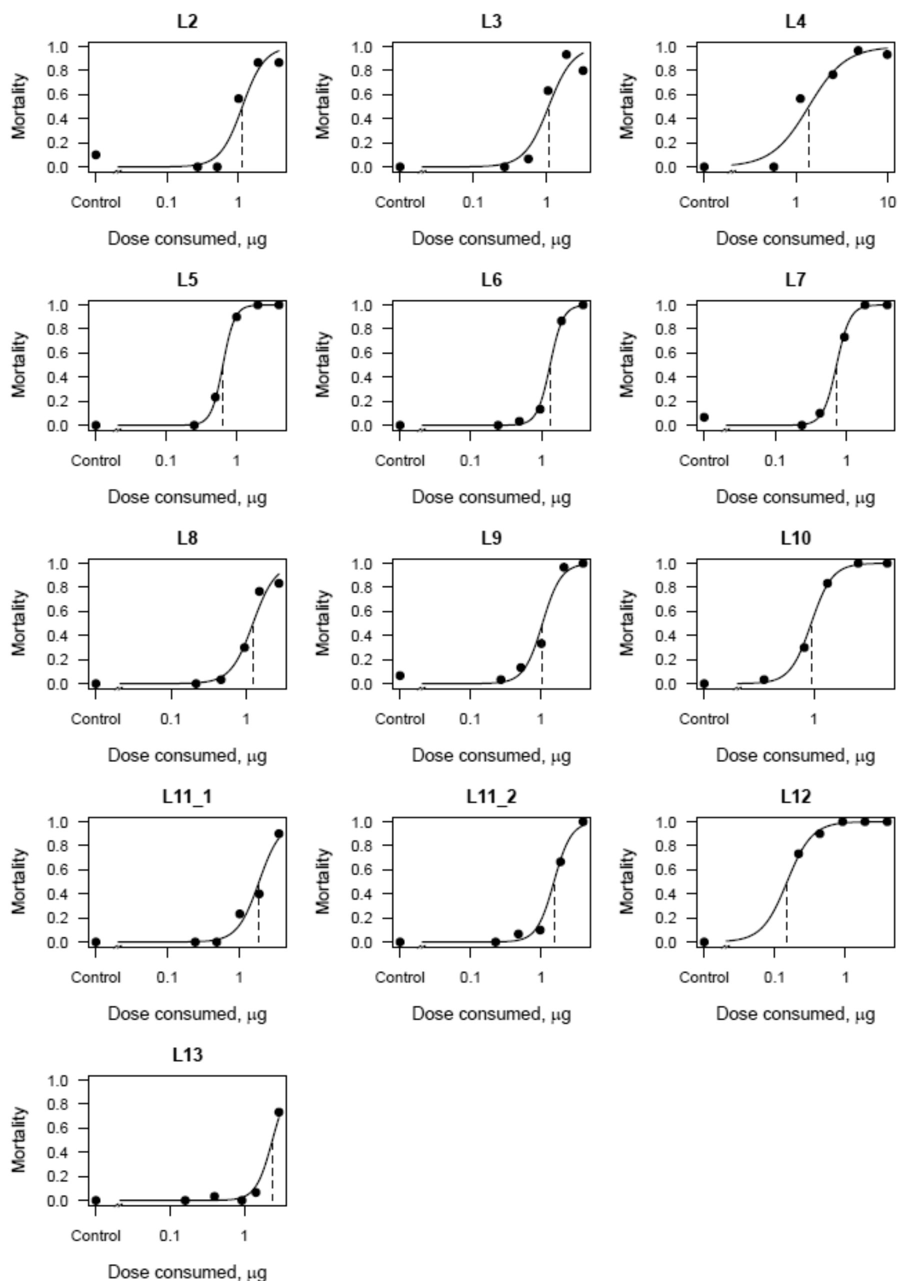

Figure 2 Dose response models fitted to the oral test data obtained at 96 h after exposure in 12 laboratories (L). The x-axis is a log scale. The dashed lines denote LD_{50}.

Although all labs performed identical tests, results of lab12 and lab13 deviated from the other participants. These two latter labs did not produce proper dose-response curves since either small and/or intermediate effects (lab12) or only large mortality (lab13) was observed. This resulted in the calculated LD_{50} values, which were either considerably lower or higher than those of the other labs (fig. 2).

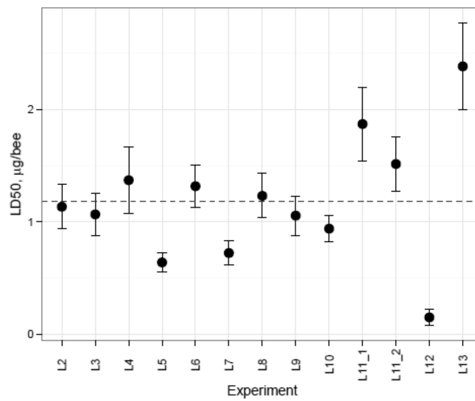

Figure 3 Inter-laboratory variation of the LD_{50} estimates obtained in the oral exposure experiments at 96 h after exposure. Vertical lines denote the 95% confidence intervals. The dashed horizontal line represents the overall mean LD_{50}.

Acute contact test – individual housing

In total 11 tests were performed by 10 laboratories (fig. 4). Note that the results of lab1 could not be included in the analysis since the control mortality exceeded 45%. The elevated mortality was traced back to a problem with the physical test set-up and was consequently considered being an artifact not related to the test design.

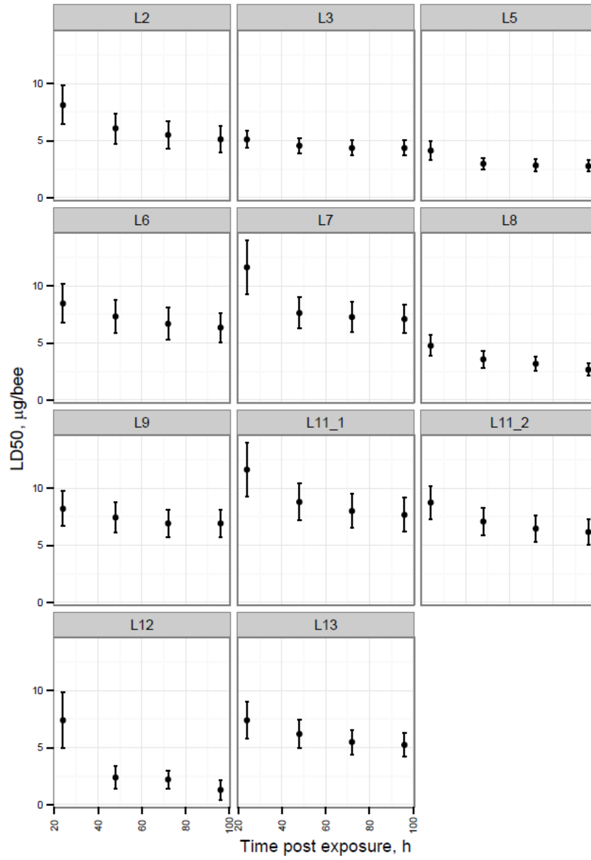

Figure 4 Point estimates of LD50 and their 95% confidence intervals obtained in the single contact tests at 24, 48, 72 and 96 h after exposure in 10 laboratories (L).

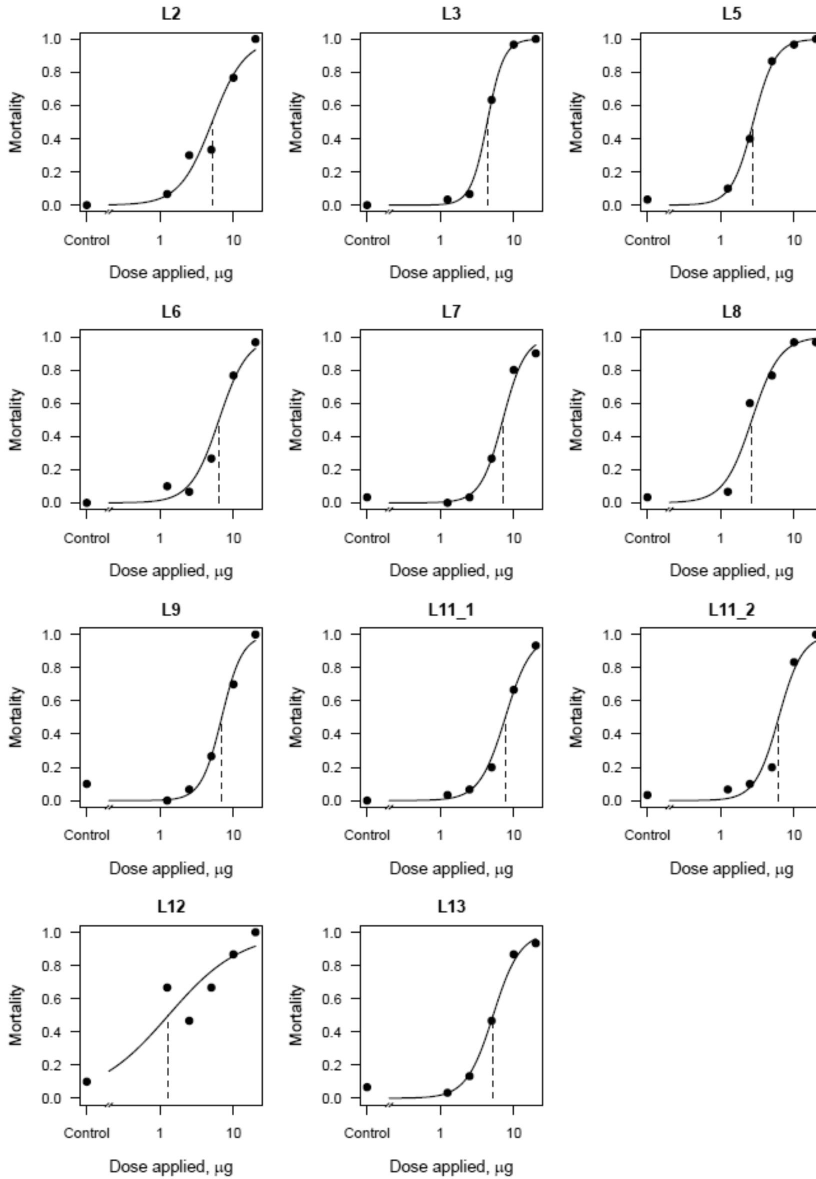

Figure 5 Dose response models fitted to the single housed contact test data recorded at 96 h after exposure in 10 laboratories (L). The x-axis is a log scale. The dashed lines denote LD_{50}.

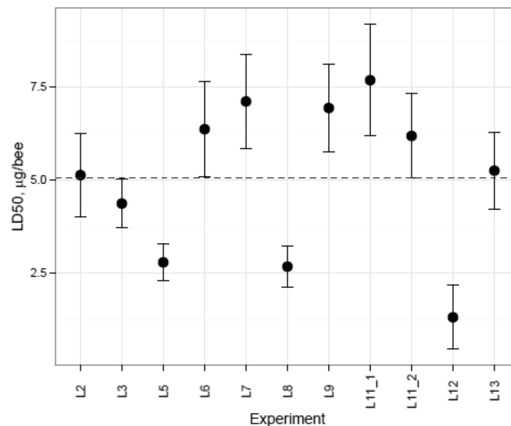

Figure 6 Inter-laboratory variation of the LD_{50} estimates obtained in the single housed contact tests at 96 h after exposure in 10 laboratories (L). Vertical lines denote the 95% confidence intervals. The dashed horizontal line is the overall mean LD_{50}.

Acute contact test – group housing

In total, 13 tests were performed by 13 laboratories. There was a considerable variation among the dose-response curves originating from different labs. Furthermore, for 3 laboratories a control mortality >10% was observed (Figure 8). The variation in response is reflected in a similarly large variation of the calculated LD_{50} values (Figure 9). It should be mentioned, however, that the application of the 5 µL droplet containing 0.5% (in most cases) Tween80 as a wetting agent did not provide the expected spread over the treated bumblebee. The droplet more or less stayed intact on the thorax and once the animals were put in the group housing boxes the droplet could either be partially or completely removed (due to the contact with other animal or with filtration paper on the bottom of the holding container). Some laboratories also reported aggressive behaviour of the bumblebees after introducing them in the group housing boxes. As the bumblebees originated from different hives, this could be due to the hierarchy fights. This could also explain the observed higher control mortalities and higher variation of LD50 values compared to the single housing.

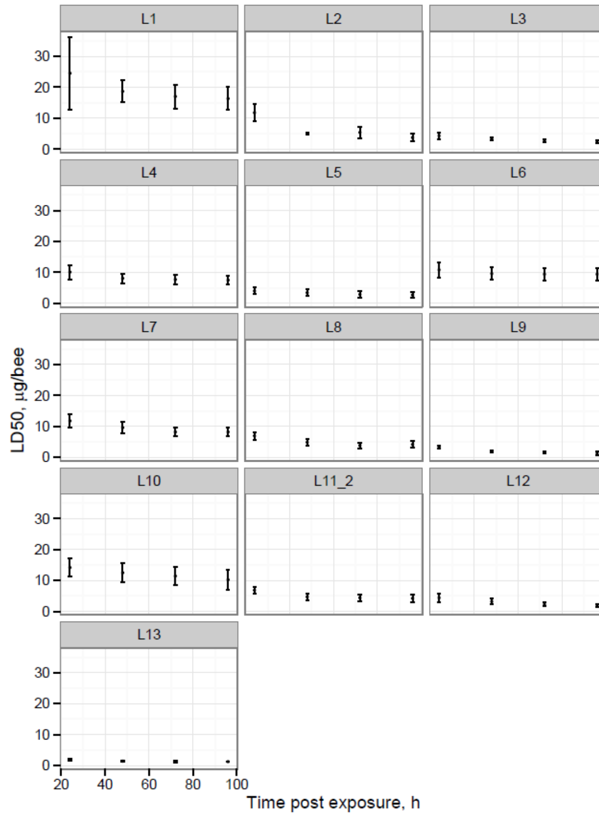

Figure 7 Point estimates of the LD50 in 13 laboratories (L) and their 95% confidence intervals obtained in the group housed contact tests at 24, 48, 72 and 96 h after exposure

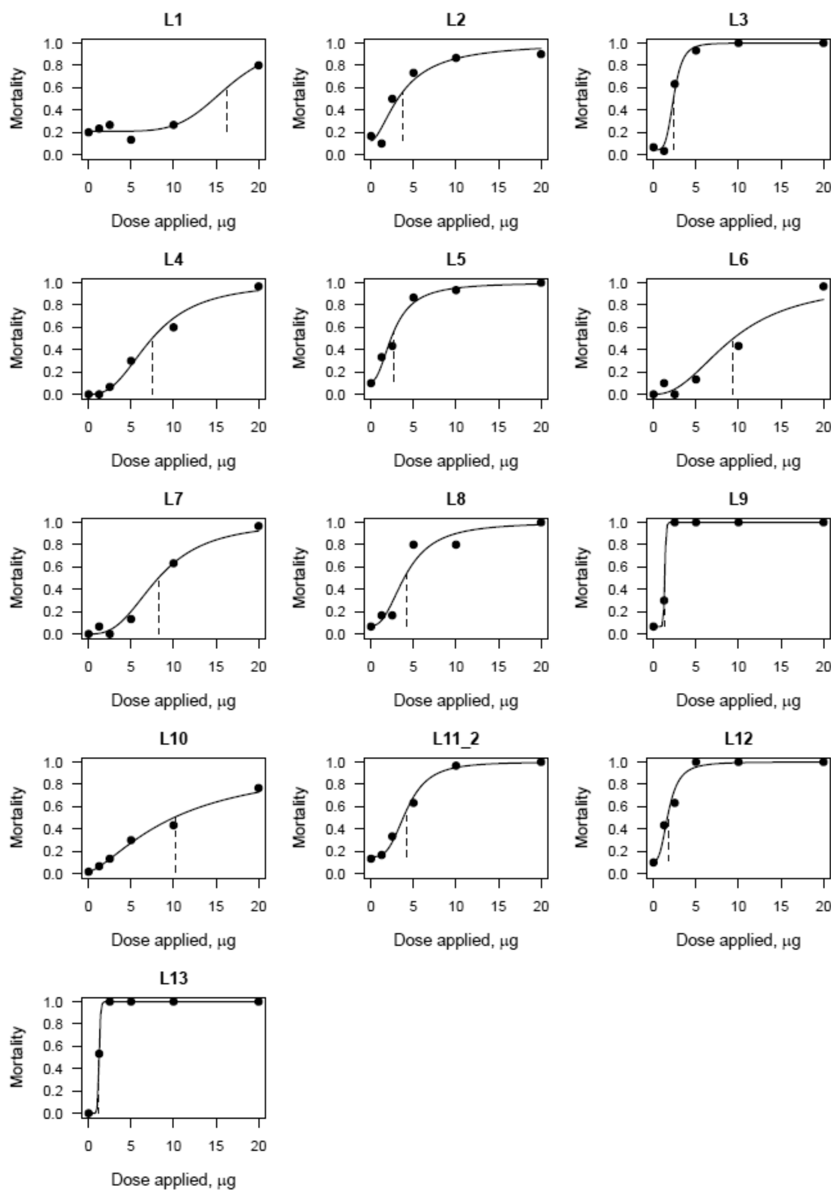

Figure 8 Dose response models fitted to the group housed contact test data obtained at 96 h after exposure in 13 laboratories (L). The x-axis is a log scale. The dashed lines denote LD_{50}. Note that in some cases these lines

do not start from the origin as the control response was non-zero.

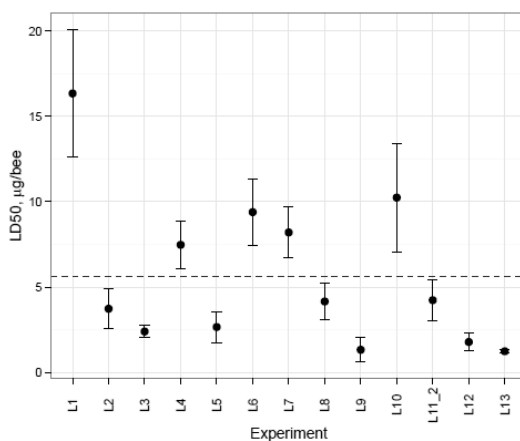

Figure 9 Inter-laboratory variation of the LD_{50} estimates obtained in the group housing contact tests at 96 h after exposure. Vertical lines denote the approximate 95% confidence intervals. The horizontal line is the overall mean LD_{50}.

Conclusions

1. In most experiments low control mortality, typically not exceeding 10%, was reported systematically, showing feasibility and reliability of the proposed methods.
2. In the group housed contact test, however, 3 out of 13 experiments did exceed 10% control mortality. Possibly, aggression between females originating from different colonies introduced additional stress, thus elevating the control mortality and suggesting the presence of a 'housing effect'.
3. Comparison of the different housing methods (individual vs group) in the contact exposure tests revealed more variable LD_{50} estimates for the group housing, whereas the endpoints obtained in the single housing experiments were more consistent among laboratories.
4. The acute oral exposure tests were found to generate slightly more variable LD_{50} estimates compared to the single housed contact tests but in no experiment control mortality exceeded 10% after 96h. The higher variability in LD_{50} estimates could possibly be explained by variation of the experimental conditions among the different laboratories (i.e. starving time, exposure time, etc.).
5. There was a tendency for decline of the LD_{50} estimates over time, which, however, would typically slow down by 48 h after exposure. This finding suggests that an experiment could potentially be stopped after 48 h and only be prolonged if mortality is increasing between 24 and 48h whilst control mortality remains at an accepted level, i.e. 10%.
6. Tween80 was found to be an unsatisfying wetting agent. Therefore, Tween 80 will not be used in further testing.

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