# Revision and adaptation of existing pollinator guidelines for testing of microbial biopesticides

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ABIM 2023
23-25 October 2023, Basel, Switzerland
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## Introduction

Currently, there is a growing interest in developing biopesticides as sustainable tools in the global plant protection market. Biopesticides are items that can control pests, but are derived from natural materials. Microbial biopesticides are one type, which are specific microorganisms that have modes of action including infectivity and pathogenicity (ability to cause disease), rather than toxicity alone, which contrasts to chemicals pesticides. However, their potential ecotoxicological risk still needs to be assessed, taking into account their mode of action and their lifecycle. Also, any dose verification techniques used need to be microbiology-based, rather than via analytical chemistry. Here, we present our methodological developments for testing microbial biopesticides on honey bees, which have been adapted from established terrestrial ecotoxicology guidelines. We first investigated bee survival with different foods, when prolonging the chronic adult honey bee test from 10 to 30 days. Secondly, we adapted the honey bee larvae test to investigate pathogenicity evaluation of a microbial pesticide through feeding. Thirdly, the bacterial quantification in the inoculated food, using the colony-forming unit (CFU) counting, was investigated.

## Materials & methods

**Chronic adult honey bee test:**
The observation period of the chronic adult honey bee test (OECD 245[1]) was extended from 10 to 30 days. This is to align with current EPA requirements (OPPTS 885.4380[2]), which is to allow sufficient time to detect the pathogenicity and infectivity of biological agents within the lifespan of an adult honey bee. However, prolonging the test may result in increased bee mortality. Different foods were tested to evaluate their effect on bee survival up to 30 days. Newly emerged worker bees were fed *ad libitum* with either 1) 50% sucrose, 2) 75% honey solution, 3) 50% sucrose solution and pollen paste or 4) 75% honey solution and pollen paste. The mortality was recorded daily.

**Honey bee larvae test:**
The pathogenicity of a commercial product containing the bacterial biopesticide *Bacillus thuringiensis aizawai* was tested on honey bee larvae through feeding. Honey bee larvae were orally exposed to different bacterial concentrations following the honey bee larval test guideline (OECD 237[3]), with a single exposure dose on day 4, and an observation period extended to the emergence of the adult bee (day 22). The mortality was recorded at the timepoints described in OECD 239[4].

**Bacterial quantification:**
The bacterial concentrations in the inoculum solutions and larval food was determined through CFU counting on agar plates. The larval food was 10-fold diluted, plated on non-selective nutrient agar and incubated at 33°C for 24 h. After the incubation period, the CFUs were counted. As the plates were non-selective, an untreated control was included to verify the absence of contamination by other microorganisms.

**Image 1.** Bacterial quantification on nutrient agar

## Results & discussion

- **Chronic adult honey bee test**
  *Ad libitum* access to pollen enhanced the survival of the adult bees over 30 days, as compared with no pollen (**Fig.1**). Survival was higher when the bees were fed with 50% sucrose compared to 75% honey. The lowest mortality rate (20%) was observed in the group fed with 50% sucrose solution and pollen. This mortality rate was above the control validity criteria of the current chronic adult honey bee test over 10 days (15%), but is low enough for statistical comparison of the survival rate. The combination of 50% sucrose solution and pollen paste is considered as a suitable food for adult honey bees in a chronic test over an observation period of 30 days.

- **Honey bee larvae test**
  The single feeding of 4-day-old honey bee larvae with *B. thuringiensis aizawai* led to mortality, which was dose-related and moderate to high mortality in all doses tested (**Fig.2**). No NOED could be determined.

- **Bacterial quantification in larval food**
  The CFU/larva counted from the inoculated food were similar to the expected CFU/larva in all except for the highest dose (**Table 1**), which was 10 times higher than expected. The reason for this could not be determined.

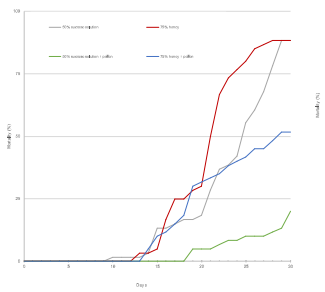

**Figure 1.** Mortality of adult honey bees maintained in laboratory and fed with different foods (n= 60 bees per condition, 6 replicates of 10 bees).

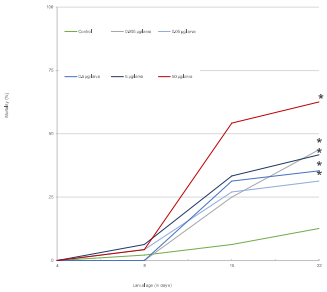

**Figure 2.** Mortality of *in vitro* reared honey bee larvae after single exposure to *Bacillus thuringiensis aizawai* at different doses (µg/larva). Asterisks represent statistically significant differences with the control group (Step-down Rao-Scott-Cochran-Armitage Test; $p < 0.050$; one-sided greater; n=48 larvae per condition, 3 replicates of 16 larvae).

| Target doses (µg Test Item/larva) | Expected CFU/larva | Counted CFU/larva |
|---|---|---|
| Control | 0 | 0 |
| 0.005 | 2.96E+02 | 2.46E+02 |
| 0.050 | 2.96E+03 | 3.30E+03 |
| 0.500 | 2.96E+04 | 3.00E+04 |
| 5.00 | 2.96E+05 | 2.70E+05 |
| 50.0 | 2.96E+06 | 2.20E+07 |

**Table 1.** Summary results of the bacterial quantification from honey bee larvae inoculated food.

## Conclusion

This work was carried out with the aim of adapting and optimising two regulatory honey bee test guidelines for microbial biopesticide testing. The observation period of the chronic honey bee adult test was prolonged to 30 days and suitable food was found to maintain the bees over this time. The honey bee larvae test was successfully adapted to evaluate the pathogenicity of a bacterial pesticide on honey bee larvae until emergence and a method of bacterial quantification in the exposure material (here larval food). The presented adaptations meet the requirements for testing microbial pesticides on honey bees under laboratory conditions.

## References

[1] OECD Guideline for the testing of chemicals No. 245: Honey bee (*Apis mellifera L.*) chronic oral toxicity test (10-day feeding); Adopted 9 October 2017.
[2] OPPTS 885.4380, Microbial Pesticide Test Guidelines, Honey Bee Testing, Tier I, EPA, February 1996.
[3] OECD Guideline for testing of chemicals No. 237: Honey bee (*Apis mellifera L.)* larval toxicity test, single exposure. Adopted 26 July 2013.
[4] OECD No. 239, Guidance document on honey bee larval toxicity test following repeated exposure, Series on Testing and Assessment, 15 July 2016.