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ECOLOGICAL RISK ASSESSMENT FOR MICROBIAL PESTICIDES

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Registering a microbial pesticide

There are only three problems to overcome:

1. The regulatory framework is inappropriate.
2. The study guidelines are inadequate.
3. Nobody can agree how to interpret the results.

Regulatory environment

- Biopesticides are regulated in Europe under (EC) No 1107/2009 in exactly the same manner as conventional agrochemicals.
- Microbial data requirements are a direct read-across from those for conventional agrochemicals.
- Some of the test species that have historically been used for chemical ecotoxicology testing are unsuitable for microorganisms.
- Where test species are suitable, the study guidelines often need to be adapted to take account of microbial modes of action.

Modes of action

Biological / physical

- Cuticle penetration.
- Dissemination in haemocoel.
- Hyphal tissue invasion and proliferation.

And / Or

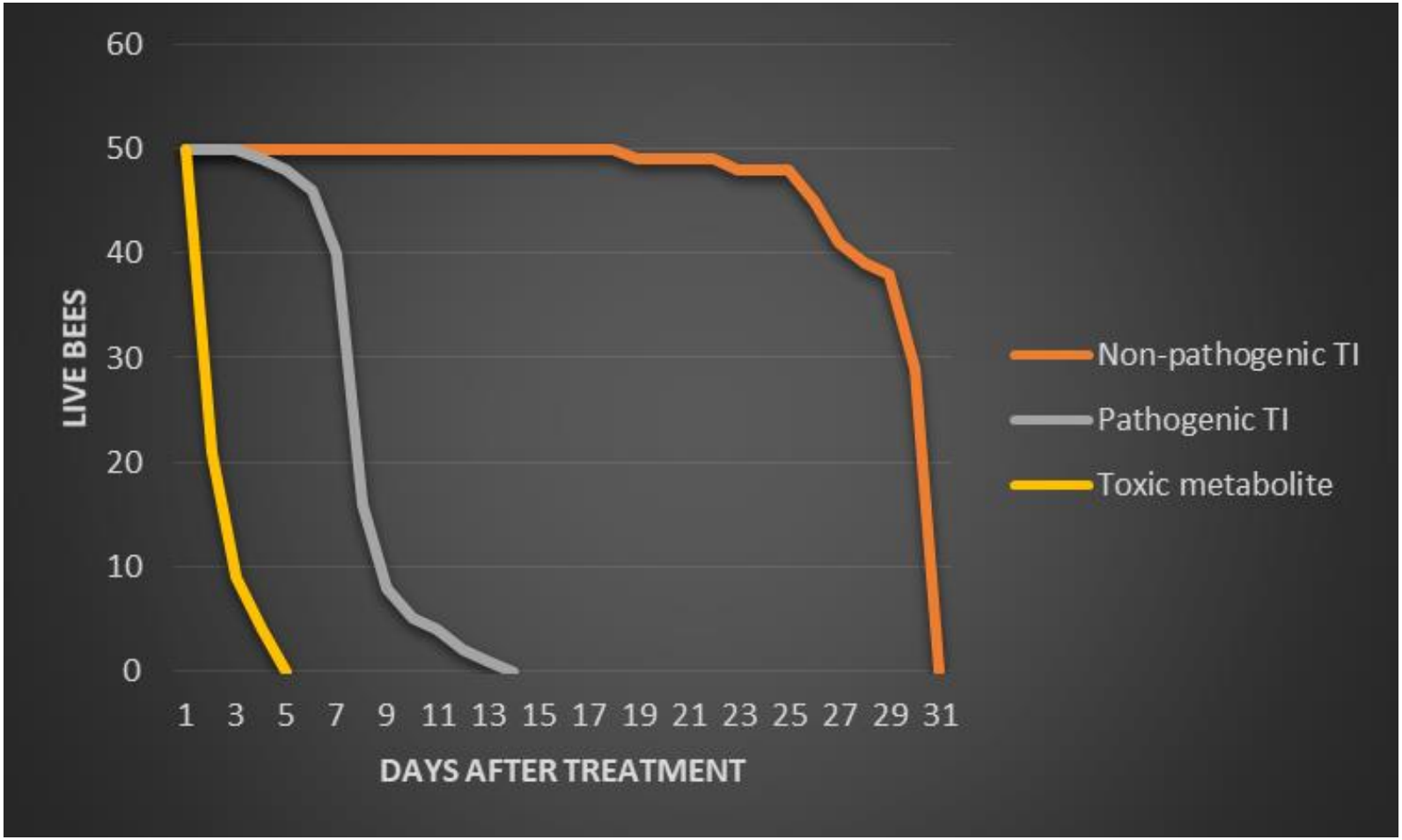
Chemical

- Endotoxins or toxic metabolites.
- Toxic manufacturing impurities.

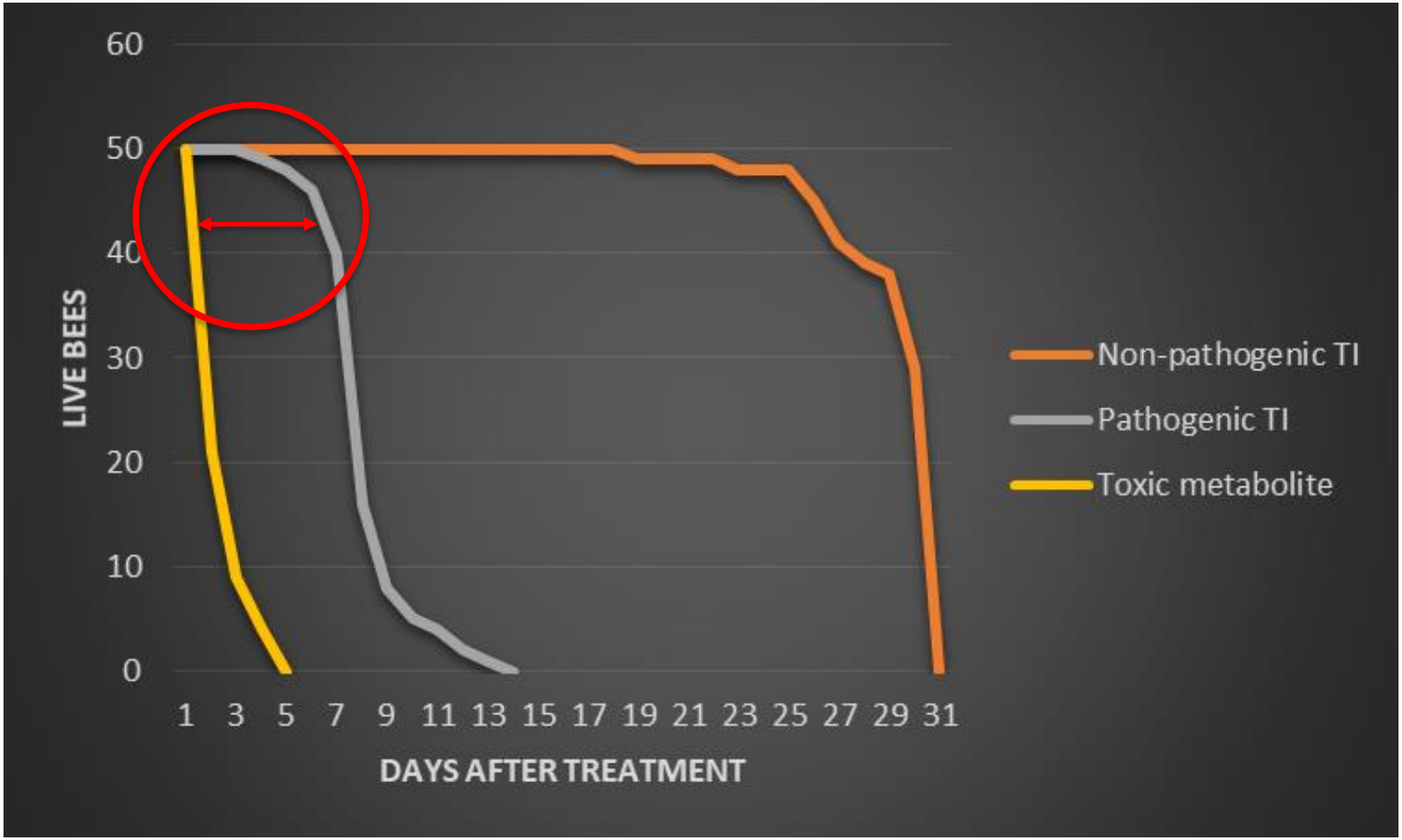
Dose response

- Ecologically, a microorganism will either be infective to a particular organism or it won't, and once the minimum effective dose has been achieved there is usually no further dose-response relationship.
- Microbial studies are generally conducted as limit tests at the Maximum Hazard Concentration, which is 10 – 100x the Maximum Field Application Rate.
- The active substance is usually tested rather than the formulation.
- The exception is aquatic studies, where the extreme hydrophobicity of spore powders makes the formulation easier to use.

Typical survival curves



Typical survival curves



Biology or chemistry?

- In common with agrochemicals, toxic manufacturing impurities or metabolites present in the TGAI are quick to manifest themselves.
- Metabolites produced during the infection process, or physical damage resulting from internal hyphal growth, typically take at least 3-5 days to show effects.
- Many standard Tier I studies are either too short to detect these effects, or the test species is unsuitable for microbial risk assessment.

Microbial study guidelines

- With no specific microbial study guidelines in Europe, applicants must either come up with their own proposals or go to a CRO with relevant experience.
- None of these methods have been validated or ring-tested for microbials.
- The US OCSPP 885 series guidelines **are** specific to microorganisms, but are severely lacking in detail compared to ESCORT / OECD guidance and are therefore of little practical value.

Some examples from standard test species

1. Springtail / collembolan (*Folsomia candida*)
2. Parasitic wasp (*Aphidius rhopalosiphi*)
3. Honeybee (*Apis mellifera*)
4. Water flee (*Daphnia magna*)

Folsomia candida (IOBC/WPRS)

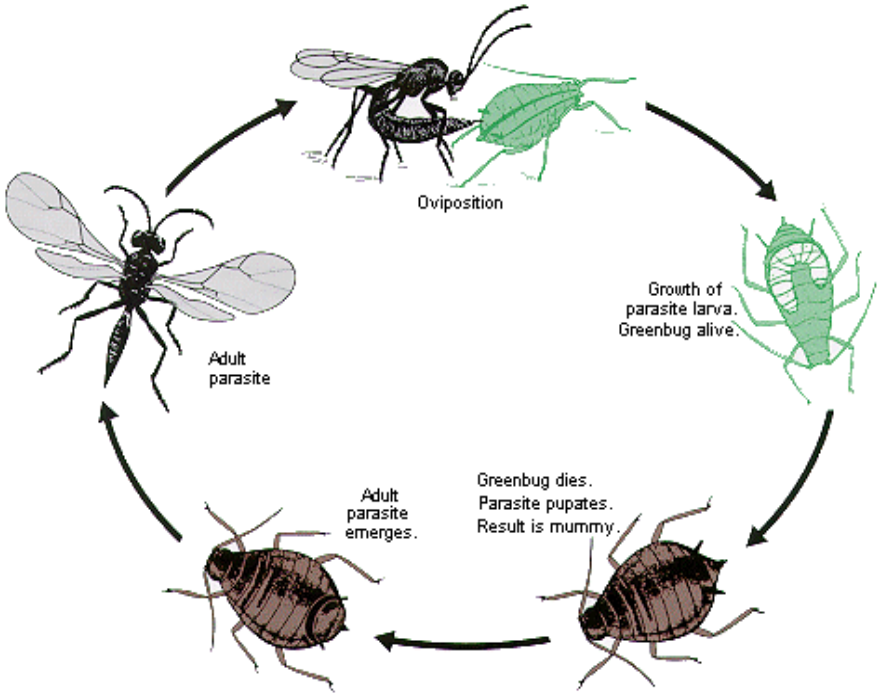
- Soil-dwelling arthropods that graze on microorganisms, including mycorrhizal fungi – an activity thought to negatively affect the symbiotic establishment on plant roots.
- Feeding experiments reveal a preference for a wide range of common saprobic fungi over mycorrhizal fungi, with a concomitant increase in fecundity.
- Collembolans are thus unlikely to be sufficiently sensitive species for microbial ecotox testing – a conclusion borne out by experience at APIS.

Aphidius rhopalosiphi (IOBC/WPRS)

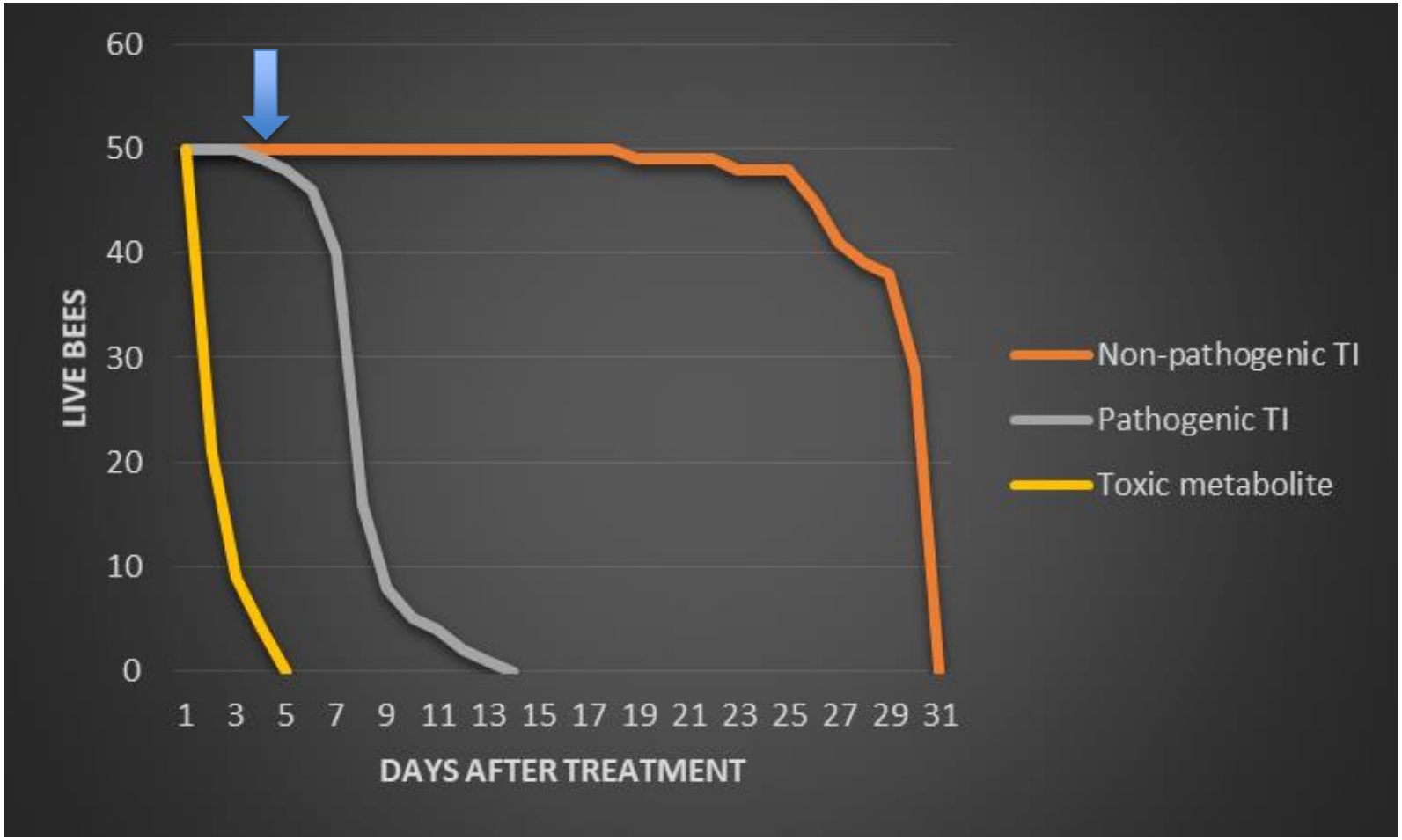
- The standard parasitic wasp species.
- Test design requires adult female wasps to be confined on treated substrate for 48 hours, after which a mortality assessment is made.
- Surviving females are then transferred individually to aphid-infested plants for 24 hours before being removed.
- Parasitism rates are assessed 10-12 days later.

Aphidius rhopalosiphi life cycle

- Female wasps mate within 24 hours of emergence.
- >90% of eggs are laid during the following three days.



Time to effect is critical



Aphidius rhopalosiphi life cycle

- Microbial pesticides rarely have directly fatal or through-the-female effects this quickly, so adverse effects on survival or fecundity are unlikely to be detected in this study.

- After the majority of eggs have been laid the cause of death – old age, predation or entomopathogen infection – is irrelevant.

- Microbial pesticides can have a negative impact on adult wasp emergence when applied to parasitized hosts, so there is invariably some collateral damage as a consequence of crop protection use.

Honeybees (OECD 213/214)

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Study design

- Young worker bees removed directly from hive and caged in groups of 10.
- Test item applied directly to thorax, or provided in 50% sucrose solution for 4-6 hours.
- Mortality observations made for 2-4 days.
- Observation period extended for microbials.

Honeybees, Tier I (OCSPP 885.4380)

Test species
Testing shall be performed on the honey bee, *Apis mellifera*.

Age
When the MPCA may be expected to affect insect larvae, test insects should include honey bee larvae.

Route of exposure
When the MPCA may be expected to act by a dietary route of exposure or are particles of such a size that they might be carried back to the hive like pollen, the honey bees must be dosed orally. Testing in the hive may be necessary.

Controls
A concurrent control group is recommended and should be treated with microbe-free (or nonviable microbe) material from the culture system used for propagation of the MPCA.

Duration of test
Control and treated bees should be observed for at least 30 days after dosing.

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Chain of unintended consequences

- Extending observation period to 30 days entails rearing bees directly out of brood frame in an incubator, not picking them out of a hive.
- Diet then needs to be optimized, as sugar water alone is insufficient for adult development.
- Diet is known to affect pesticide resistance, and including pollen changes the expression of genes relating to detoxification.
- LD_{50} values for toxic reference substances may then stray outside the study validity criteria.

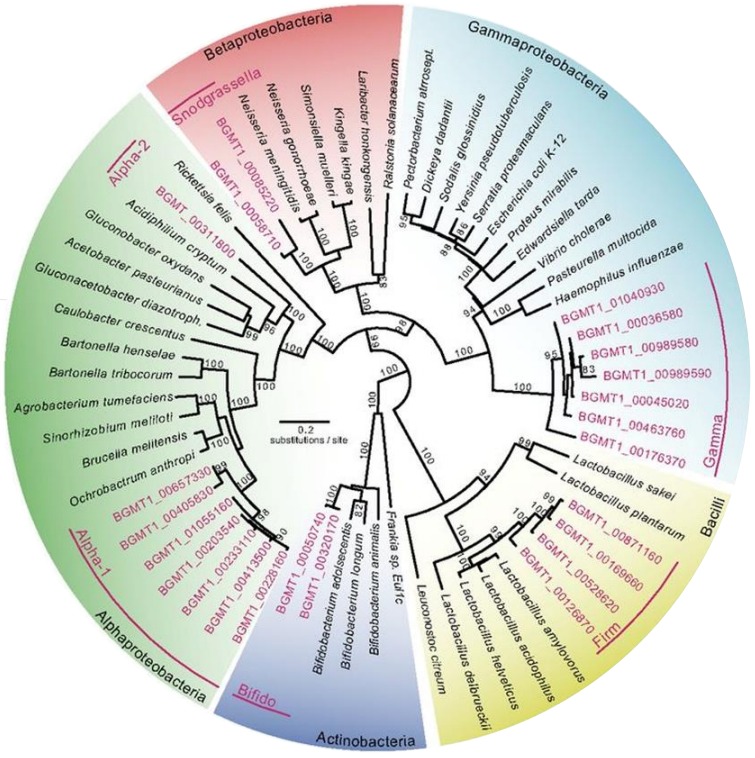
A more fundamental problem

Are we even asking the right questions?

- The current test designs are completely irrelevant to managed honeybee colonies.
- Honeybees exhibit extreme sociality, living in high density colonies of several thousand individuals.
- High-density living favours pathogen spread, in response to which honeybees have evolved multi-layered defences, from colony-scale mechanisms down to individual immune responses.

A more fundamental problem

- Bees are not microbially sterile.
- Immune status is modified by commensal flora and their interactions with xenobiotic compounds.
- Some commensal gut bacteria can confer resistance to microbial infections.
- Individual immune status can show density-dependent plasticity.



Honeybee immune responses

Colony-level mechanisms:

- Removal of infected nest-mates.
- Self-removal when infected.
- Depositing corpses outside foraging range.
- Collection of antiseptic saps.
- Production of antiseptic enzymes (glucose oxidase).
- Brood fever (raising colony temperature to combat infection).

Honeybee immune responses

Individual-level mechanisms:

- Cellular responses (phagocytosis, encapsulation).
- Humoral responses (prophenoloxidase cascade → melanisation).
- The expression of some genes relating to these responses has been shown to change in response to pesticide exposure, although inconsistently.

What are the right questions?

Does the test item have the capacity to be pathogenic in lab studies?

If it does:

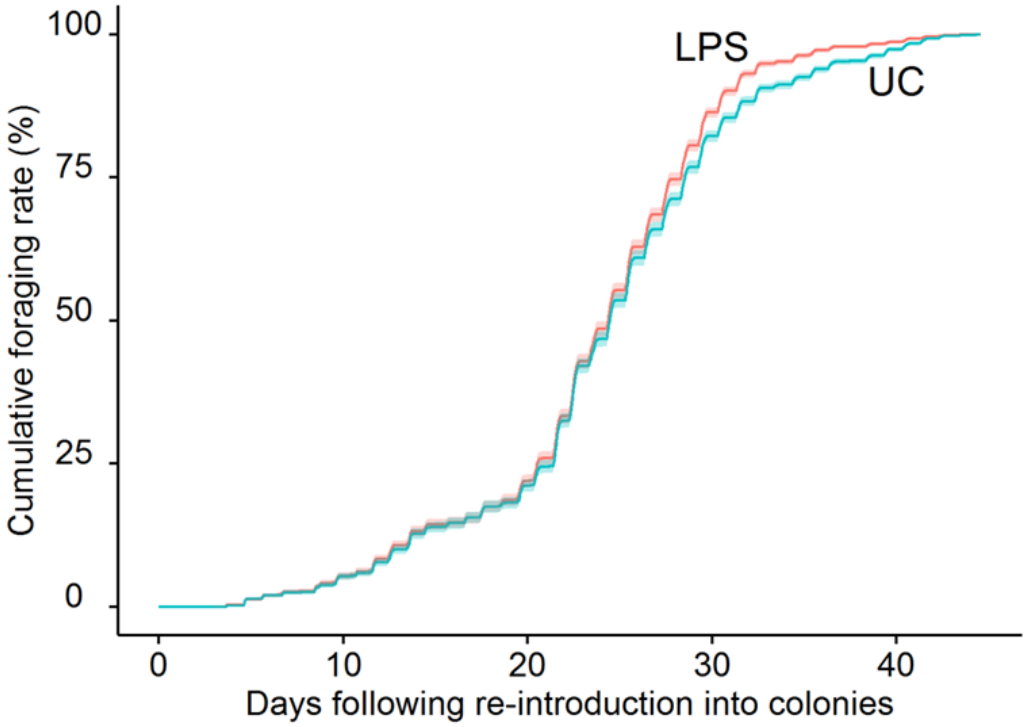
- How can we show that the test item was the cause of death?
- What effects are there in field colonies under natural conditions?

If not:

- Does it have non-pathogenic effects on colony health?

RFID tracking following LPS injection

Immune stimulation by lipopolysaccharide injection results in increased foraging intensity.



Jones, B.D. (2016) Strategies of disease resistance in the superorganism: investigations into the effects of diet on the immunocompetence and behaviour of honey bees.

Tracking immune response

- There is a trade-off between investment in immune response and longevity.
- A heavy investment in immune function, such as might be triggered by microbial invasion, can lead to a shorter lifespan, and would also be triggered by the inactivated test item.
- A significant foreshortening of longevity in the inactivated treatment group, coupled with an increase in antimicrobial peptides, would suggest a mechanism other than direct pathogenicity.

Daphnia magna (OECD 202)

- *Daphnia magna* is a useful indicator of the presence of toxic metabolites or manufacturing impurities when exposed to sterile filtrates, but is also extremely sensitive to environmental stressors such as suspended particulate matter.
- Testing the TGAI invariably leads to rapid mortality, and this is always ascribed to the physical nature of the test item rather than to any intrinsic pathogenicity.
- Extreme hydrophobicity of most microbial test items often necessitates adjuvants at levels that would be immediately fatal to *Daphnia*.

Dose verification in aquatic studies

"If any contract lab provides accurate dose verification from an aquatic study my first assumption would be that they'd made it up."

– Ecotox evaluator from leading EU regulatory authority

- Microbial dose verification in aquatic studies is notoriously difficult due to the extreme hydrophobicity of microbial test items.
- Even if the formulation is tested, getting a homogenous distribution throughout the media is almost impossible, leading to wildly inaccurate results.
- Enumerating aged media is complicated by microbial contamination.

Considerations for ecotox testing

- Microbial pesticides show strain-dependent variation in host specificity, and consequently also strain-dependent non-target effects.
- Some of these effects are due to direct toxicity from metabolites or manufacturing impurities, whilst others are due to pathogenicity.
- Microbial ecotox testing needs to address the biology of microbial test items and the selection of suitably susceptible test organisms.
- An opportunity to re-think the ecological risk assessment of microbial pesticides.

Current activities

APIS is involved in two current projects to improve the testing guidelines for microbial pesticides:

- EPA / BPIA project on improvement of OCSPP microbial testing guidelines.
    - This covers all studies, including mammalian toxicology, but the immediate focus is on NTAs.
- ICP-PR Working Group on the impact of microbial pesticides to bees.
    - Led by CTGB and EPA.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