ctgb

Datum: 26 maart 2014
Opsteller: 5.1.2.e 5.1.2.e / 5.1.2.e
5.1.2.e
Vorige bespreking: C-257.I.13

Akkoord secretaris:

C-263.I.6 **Herbeoordeling risico voor bijen van middelen op basis van thiacloprid en acetamiprid – fase IB: Synergisme**

Doel notitie
Het doel van deze notitie is het College te adviseren over het vervolgtraject van het project 'herbeoordeling risico voor bijen van thiacloprid en acetamiprid". In de eerste fase van het project is besloten dat er een tussenfase over synergisme zou worden ingelast in het project. Deze notitie gaat over de tussenfase (Collegenotitie C-257.I.13. ging over fase I), waarin is beoordeeld op welke wijze er een prioriteitenlijst van werkzame stoffen met synergistische interacties kan worden opgesteld. Daarnaast wordt er een advies gegeven of een versnelde herbeoordeling van middelen op basis van acetamiprid en thiacloprid noodzakelijk wordt geacht.

Kader
Bij het AO overleg heeft de STAS de kamer toegezegd het Ctgb te vragen thiacloprid en acetamiprid nationaal versneld te bezien met het oog op het risico voor bijen. Het doel van het project is concrete risico's van middelen op basis van thiacloprid en acetamiprid voor bijen aanpakken vooruitlopend op de herbeoordeling en besluitvorming in Europa. Het betreft 7 middelen op basis van thiacloprid en 1 middel op basis van acetamiprid.

In de eerste fase zijn veel nieuwe studies bij het Ctgb beschikbaar gekomen aangaande thiacloprid en acetamiprid en het risico voor bijen. De nieuwe studies waarin het effect op bijen van de individuele stoffen is onderzocht, geven geen directe aanleiding om versneld te gaan herbeoordelen. Echter, de studies waarin het effect op bijen van combinaties aan stoffen is onderzocht tonen aan dat de toxiciteit voor bijen mogelijk wordt verhoogd als thiacloprid of acetamiprid worden toegepast in combinatie met andere gewasbeschermingsmiddelen.

Op basis van openbare literatuur en de EFSA scientific contribution over bijen van 2012 blijkt dat neonicotinoïden niet de enige groep stoffen is waar synergisme optreedt. Daarom is er een tussenfase (fase IB) in het project gelast, waarin wordt onderzocht in hoeverre andere werkzame stoffen ook synergisme vertonen, zie ook collegenotitie C-257.I.13.

In deze tussenfase wordt het College gevraagd om besluitvorming te maken over de volgende aspecten aangaande het vervolgtraject van het project:

1. op welke wijze er een prioriteitenlijst van werkzame stoffen met synergistische interacties moet worden opgesteld.
2. besluiten of een versnelde herbeoordeling van middelen op basis van acetamiprid en thiacloprid noodzakelijk wordt geacht.
3. vervolgtraject richting EFSA en Europese herbeoordeling van de stoffen acetamiprid en thiacloprid

Er zijn afspraken gemaakt met het ministerie dat wij hen in april informeren over de uitkomst van fase IB.

recente ontwikkelingen
Er zijn18 maart twee moties aangenomen waarin de tweede kamer de regering verzoekt een moratorium in te stellen op het gebruik van neonicotinoïden (motie 27858-125) en een nationaal traject in gang te zetten voor een volledig nationaal moratorium op alle neonicotinoïden en op fipronil (motie 27858-155).

Introduction
Bees are important pollinators of both managed crops and wild flora. Honey bees can forage over a large area, with a mean radius of 5 km from their hive giving a potential foraging surface of over 7500 ha. This increases the chance of being exposed to different stressors, eg. xenobiotics like pesticides. Given the complicated structure of a honey bee colony, this may not only pose a risk for the individual bee, but for the whole hive. Polluted pollen, nectar etc can be taken back to the hive, where a combination of all these products from different sources can expose the bees to a mixture of a wide range of xenobiotics (an overview of active ingredients from ppp's found in bee products is given in appendix III, however care must be taken, often the number of active ingredients analysed per sample is limited to the ingredient under investigation). Recently, in the USA one hundred and twenty one different pesticides and metabolites were identified in the hive with an average of seven pesticides per pollen sample (Mullin et al. 2010; Johnson et al. 2010). In other words, bees are being exposed to a complex mixture of xenobiotics outside as well as inside the hive. Unfortunately, not much is known about the effects on bee fitness. However, the combined effect of consecutive or simultaneous exposure to mixtures of xenobiotics has been described for honey bees in scientific literature. Effects of mostly binary mixtures of plant protection products have been assessed and, depending on the concentration, the combination, the life stage of the bees and the exposure strategies, were found to be either additive, synergistic or antagonistic.

Over 100-fold increases in honey bee toxicity of the neonicotinoid insecticides acetamiprid and thiacloprid were described by Iwasa *et al.* 2004 when combined with piperonyl butoxide, triflumizole and propiconazole. Such major synergistic interactions are rare, but can not be ignored. Especially since these active ingredients tested in the study are authorized in over 100 of the same crops (indoor and outdoor uses) (submitted by Tegelaar and van der Sluijs, 2013).

However it was recognized that it should be assessed if there are other potential pesticide combinations which have synergistic interactions before one can solely focus on the synergistic interactions of thiacloprid and acetamiprid. Therefore public literature and guidelines were studied to:

1. investigate the occurrence of synergistic mixture interactions described for plant protection products (ppp's) and honey bees, and the presence of their residues in bee products.
2. assess the feasibility of predicting synergistic mixture interactions with single substance information.
3. assess developments in other areas, including possible promising approaches for chemical screening for prediction of synergistic interactions.

Part A of this paper describes the outcome of these investigations. In Part B the conclusions from Part A are applied to the case of thiacloprid and acetamiprid.

PART A

Observed synergy in bee studies

In Appendix III table 1 an overview is given of the active ingredients that were found to be involved in some type of synergistic mixture interaction, with at least a 3-fold increase in toxicity for the measured endpoint in honeybees. In addition, their presence in bee products is indicated as well. Unfortunately, no general conclusions could be made on the likelihood of an active ingredient being involved in a synergistic mixture interaction. However, a few trends were observed:

- Insecticides seem to interact with classic synergists (PBO)
- Insecticides are found in interactions with fungicides
- Neonicotinoids seem to interact with EBI fungicides
- Lambda-cyhalothrin often seems to be involved in mixture interactions

Care must be taken with these trends, as the enormous number of possible binary mixtures have not all been tested. In addition, the choice of active ingredients that have been tested can be biased, either by being known to be involved in interactions via other studies, or because the active ingredient is interesting for economic or environmental reasons and hence chosen more often than other active ingredients. In addition, most results come from laboratory studies using very high exposure concentrations which do not reflect 'real life situations'. In Appendix III table 2.15 results for semi-field studies are given, executed for a limited amount of active ingredients. Here no mixture interactions were found. However, field studies are limited: Their reproducibility is variable, due to changing environmental conditions in the foraging area, which can extend up to a 9 km radius around the hive. Observations made in a particular field experiment might not be representative of the range of effects that could occur under real conditions (Vd Sluijs *et al.* 2013).

Overall, the toxicity data could not be used to generate general conclusions on predicting synergistic interactions between chemicals on bee toxicity.

Predicting mixture interactions

In order to minimize the amount of testing performed and avoid testing all possible mixture combinations, there is a strong need to predict possible mixture effects and particularly the potential for synergistic effects.

A necessary step in evaluating the risk of exposure to multiple known xenobiotics and, hence, mixture toxicity, is to make a prediction of the expected mixture effects. The concepts of Concentration Addition (CA)) and Independent Action (IA, also termed Response Addition) allow valid calculations of expected effects if the toxicity of the individual mixture components are known.

CA assumes similar action of the chemicals in the mixture, while Independent Action takes dissimilar action as the starting point. In practice, this means that CA is used as the reference when testing chemicals with the same or similar modes of action, while Independent Action is the preferred reference model in the case of chemicals with different modes of action.

For a given mixture, the observed effect can deviate from the predicted mixture effect.
As elaborated in the supporting information Appendix II, CA seems to describe ecotoxicological mixture effects reasonably well, but none of the reference models can be used to predict interactions. Attempts have been made to investigate the underlying mechanism of synergistic mixture interactions, and combinations of, for instance, an insecticide with a classic synergist often trigger effects greater than the sum of their individual effects. Unfortunately for the majority of possible mixture combinations it still remains (an educated) guess as to what mechanism results in the observed synergistic effect. A short list of considerations about a potential mechanisms for toxicologically significant synergistic effects is given in the SCHER opinion (2012) :

- Can one or more components significantly enhance the uptake of other components?
- Can one or more components inhibit significantly the excretion/clearance of other components?
- Do one or more of the components exert toxic action via the formulation of an active metabolite(s) and might one or more of the components induce the drug metabolizing enzymes that may be involved in the formation of these active metabolite(s)?
- Can two or more components act on different enzymes in an important metabolic pathway?
- Can two or more components act on different elements of cellular protection mechanisms or cellular repair mechanisms?

As becomes clear from the list above, detailed information on the toxic mode of action (MoA) of the xenobiotics involved is required to assess an interaction potential..

Mode of action as a tool for predicting synergistic interactions
MoA refers to the type of response in an exposed organism or to the critical steps or features of the mechanism required for the particular biological response. MoA should consider some aspects of the critical biochemical pathway and the resulting physiological and behavioural changes produced by alterations in that pathway by the toxic agent (Borgert et al. 2004). As postulated MoA, then, is a biologically plausible sequence of 'key events' leading to an observed effect supported by robust experimental observations and mechanistic data (Meek 2009).

In the supporting information Appendix II-I, an overview of the proposed mechanism of the synergistic interaction, based on the individual MoA by Glavan (2013) is given. Especially with the known P450 inhibiting substances, the presumed synergistic MoA is believed to take place via inhibition of the detoxifying enzymes. This is often seen as the biochemical mechanism of the synergistic interaction between insecticides and fungicides. However, some of the same active substances are also involved in completely unidentified synergistic interactions (even though the MoA of the individual compounds for the target organism is more or less known).

With the present knowledge, a lot of crucial information is simply lacking in order to predict synergism. Major omissions and complicating factors are described thoroughly in the supporting information (Appendix II). A short list of the knowledge gaps includes:

- Knowledge of the MoA of the individual components, including dose-response information is lacking.
- Endpoints that in human toxicology often refer to a specific target organ cannot be used in ecotoxicology.
- MoA of chemicals is often not the same for different types of organisms and the knowledge about the MoA is usually poor.
- Ecotoxicological endpoints are relatively broad and are related to ecologically-relevant parameters (eg. population-relevant mortality, reproduction, biomass) instead of a specific target organ.
- Potential for synergism increases from the cellular level onwards, because interferences with uptake processes and metabolic steps may come into play.
- Different endpoints may show different mixture effects.
- Mixture interactions can result in effects in many different areas within an ecosystem (for instance prey-predator relationships).
- Mixture effects vary according to the relevant dose level, route of exposure, timing, life stage, endpoint and exposure duration of the biological target.

Due to the knowledge gaps and lack of predictability, MoA can not yet be used to prioritize chemicals for their potential to cause synergistic interactions.

Future developments in potential techniques
Even though current techniques are not suitable (yet) to predict synergy and, hence construct a sound priority list, we have assessed the developments in other areas of concern which could provide potential solutions or a way forward.

USEPA and predicting endocrine disruption potential of chemicals
The US EPA is constructing a list of potential endocrine disrupters (for humans), based on a tiered approach. Since ALL pesticides must eventually be screened, the first draft list was composed on *exposure potential* (through multiple routes), but not on potential for endocrine disruption. Chemicals could then be excluded based for instance on their molecular weight. Recently, the list generated for tier 1 has been renewed and this time is at least partially based on data from ToxCAST (***http://www.epa.gov/ncct/toxcast/***). ToxCAST uses chemical screening technologies (high-throughput screening assays) to expose living cells or isolated proteins to chemicals. These may in turn suggest potential toxic effects and eventually potential adverse health effects.
In Tier 1 chemicals were 'screened' for their potential to interact with hormone systems and Tier 2 will be about 'testing'. Currently the tier 1 screening consists of battery of 11 assays that have been developed and validated. Results have been (and are being) evaluated using a weight of evidence approach. Chemicals which via the Tier 1 tests and weight of evidence evaluation appear to have an endocrine disruption potential are then required to move to Tier 2 testing. Tier 2 is expected to involve more comprehensive studies across taxa to quantify dose-response relationships.

As for potential endocrine disrupters, a battery of screening systems could be an option for constructing a list of chemicals with synergistic interactions. In screening for interaction potential, few testing protocols are available. However, for both human and environmental toxicology, more methodologies to screen substances for their MoA are being developed, that may become feasible to identify interaction potentials.

MDR/MXR
Multi-Drug Resistance (MDR, or also often called MXR (Xenobiotics)) is mediated by membrane-based transporters which recognize a wide variety of chemical structures (including xenobiotics) as substrates and pumping them out of the cell, thus keeping their levels in cells low. MDR inhibitors (chemosensitizers) disrupt these transporter activities allowing xenobiotics to accumulate inside the cell.
MDR transporters are relatively unstudied in insects, but have been proposed as mechanism-based strategy to understand the impact of exposure to combined residues in honey bees (Hawthorne and Dively, 2011). After exposure to an inhibitor, exposure to acaricides or insecticides caused an increase in toxicity to bees.

Recently Campos *et al*. 2014 published first evidence for a toxic defence-based MXR mechanism in Daphnids. Exposure to binary mixtures and inhibitors were defined and predictions of the combined toxic effects were made. They hypothesised that pairings of substrate and inhibitor compounds that interact with the same efflux transporter type had joint toxic effects greater than additive (CA or IA) because interference of inhibitors of a specific transporter type will result in increased uptake and toxic effects of the respective substance. However, variability between the replicates was very high, and time-dependent effects were found. When testing the following combinations: substrate/inhibitor, substrate/substrate, inhibitor/inhibitor, in 9 out of 19 cases results indicated greater than additive effects from the binary mixtures. So also greater than additive effects were found for compounds with the same mechanism (like inhibitor/inhibitor). This system might be not selective enough to use as a screening method. In addition, if these synergistic effects could be linked to ecologically relevant endpoints (like growth, reproduction) remains unclear. A similar conclusion was made by SCHER (2012) and the report of the US National Academy's Standing Committee on Use of Emerging Science for Environmental Health Decisions, as they concluded that 'many challenges remain to be addressed before the findings from high-throughput screens and *in silico* models may be considered sufficiently robust and informative'. However, new developments like ToxCAST show their potential and considerable contribution.

Main conclusions and recommendations
- At the moment, MoA cannot yet be used to predict synergistic reactions and thus cannot be used to prioritize chemicals to further test for synergetic interactions. Alternative methods, like screening tests, are also currently not feasible to use to predict synergetic interactions.
- Because synergy can not be predicted, prioritizing chemicals according to their interaction potential can only be done based on the available mixture toxicity data.

Therefore we propose that EFSA will be asked to

1. make a list of known synergistic combinations from (public) literature and set data requirements for applicants to address the synergistic reaction
2. build a database with all available information concerning an active substance (persistence, dispersal, ecotoxicogical data, MoA, known interactions, etc.) should be built in order to gain insight into the toxic behaviour of an active ingredient. Both single substance risk assessment as well as multi-chemical risk assessment will benefit greatly from this.

With this database, MoA might be a more useful tool in future prioritizations of substances for synergetic interactions.

Other recommendations for ECHA

3. With relatively minor adjustments, much more information can be gained from toxicity tests that are already in the data requirements (like observations made in time, prolonged exposure, incipient LC50-estimates, , slope parameters, in order to get 'time independent' parameters, which are more suitable for modelling, extrapolation and weight of evidence).
4. Assess if alternatives, like the development of screening assays for interactive potential, should also be included in future prioritizations of substances for synergetic interactions.

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PART B: Acetamiprid and thiacloprid

Acetamiprid and thiacloprid will likely be on the list of chemicals known to be involved in synergistic interactions (based on (public) literature) still to be made by EFSA. The combination of EBI fungicides and neonicotinoïdes have also already been identified in the EFSA report of 2011 as having potential synergistic effects. This was confirmed by the study of Iwasa *et al* (2004), who intentionally chose these combinations based on previous mixture studies with pyrethroids. Results showed a more than 100-fold increase in the toxicity (to honey bees) of the neonicotinoid insecticides acetamiprid and thiacloprid when combined with piperonyl butoxide, triflumizole and propiconazole.

It must be noted that Iwasa's study design was indented to trigger synergistic interactions in order to confirm the mechanism behind the differential toxicity of the different neonicotinoids in honey bees. Imidacloprid is much more toxic to honey bees than acetamiprid and thiacloprid. It was never intended that the results be interpreted for a mixture effects assessment. Acetamiprid and thiacloprid are both neonicotinoids, which can be subdivided into the cyano-neonicotinoids and the nitro-neonicotinoids. Both acetamiprid and thiacloprid are members of the first group. Their lower toxicity to honey bees was attributed to their rapid transformation and the existence of different of different nAChRsubtypes (Suchail et al. 2004a, b; Brunet et al. 2005, Jones et al. 2006). Imidazole/triazole fungicides are believed to inhibit cytochrome P450systems. In a combination with acetamiprid or thiacloprid, this inhibition may prevent the biotransformation, and hence detoxification, of the insecticides thus making them more toxic. This mechanism was confirmed by Iwasa (2004) and strengthened by the lack of synergism found for the nitro-neonicotinoid imidacloprid when combined with the same fungicides.

Despite the above considerations, the occurrence of these major synergistic interactions, whether or not intentionally induced, cannot be ignored. Especially since the active ingredients tested in the study are authorized in over 100 of the same crops (indoor and outdoor uses) (submitted by 5.1.2.e 2013).

However, as noted before, with lower exposure concentrations the likelihood of severe synergistic interactions decreases. In addition, similar experiments have been carried out by Schmuck *et al*. 2003 who combined thiacloprid with various fungicides, including the EBI fungicide tebuconazole. Under laboratory conditions a 23-fold increase in thiacloprid toxicity was found, however hive vitality was not affected at the recommended use rates of the tank mix in a semi-field test.

This latter finding is confirmed by unpublished studies submitted by Bayer for thiacloprid see also , Board document C-257.I.13. In semi-field studies, honey bees were exposed during full flowering to more realistic exposure concentrations of thiacloprid simultaneously with either prothioconazole or tebuconazole. Even tertiary mixtures were tested by addition of lambda-cyhalothrin or alphacypermethrin. In these studies, no synergistic interactions between thiacloprid and fungicides were observed.
This does support that the actual synergistic interaction in the field is much lower than in the laboratory study when more realistic concentrations are used.

Even though the studies mentioned above indicate that the occurrence of synergistic effects in the field are less likely than in the lab, the exact combination tested by Iwasa *et al.* (2004) that triggered the 1141-fold increase in toxicity of thiacloprid has not been tested in a more realistic exposure regime. In addition, synergistic interactions at practical field rates have been described for deltamethrin and prochloraz in a lab study (Colin and Belzunces, 1992).

They concluded that the hypotheses about the mode of action of the synergy between prochloraz and deltamethrin might not seem as simple as inhibition of the fungicide on the oxidative metabolism of the insecticide. So, since currently no other methods are available, data regarding the mixture toxicity of acetamiprid or thiacloprid together with either triflumizole or propiconazole for honey bees needs to be assessed in relevant exposure concentrations, preferably in a controlled laboratory experiment. If no synergistic interactions are found at relevant exposure concentrations, there is no need for an elaborate field study, for which limitations and uncertainties abound. If large interactions are still detected, further testing is necessary.

WUR was consulted to propose a draft experimental design to assess possible mixture interactions of the neonicotinoids with the fungicides under investigation in the laboratory in a dose response design at relevant more realistic field exposure concentration, the draft design is given in Appendix IV.

Recommendation for the Board regarding acetamiprid and thiacloprid:
The above field studies support that no immediate national reassessment is required for plant protection products based on acetamiprid and thiacloprid. Synergy should however be included in the risk assessment in the upcoming review of the active substance dossier of acetamiprid and thiacloprid. NL is RMS for acetamiprid and we will inform the RMS of thiacloprid of our recommendations to ECHA.

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Bijlage I Directeurenbrief aan Ministerie van Economische Zaken na afronding fase I project.

Datum	30 september 2013
Betreft	Herbeoordeling risico voor bijen van middelen op basis van thiacloprid en acetamiprid

Geachte heer 5.1.2.e

Fase I van het herbeoordelingsproject over de risico voor bijen van middelen op basis van thiacloprid en acetamiprid is afgerond. In deze fase is er een analyse gemaakt van nieuwe wetenschappelijk inzichten die aanleiding kunnen geven voor een herbeoordeling van de middelen. Hierbij rapporteren we u onze bevindingen.

Er zijn veel nieuwe studies bij het Ctgb beschikbaar gekomen aangaande thiacloprid en acetamiprid en het risico voor bijen. De nieuwe studies waarin het effect op bijen van de individuele stoffen is onderzocht, geven geen directe aanleiding om versneld te gaan herbeoordelen. Echter, de studies waarin het effect op bijen van combinaties aan stoffen is onderzocht tonen aan dat de toxiciteit voor bijen mogelijk wordt verhoogd als thiacloprid of acetamiprid worden toegepast in combinatie met andere gewasbeschermingsmiddelen.

In principe wordt het potentieel effect van synergisme in risicobeoordelingen meegenomen als onderdeel van de generieke veiligheidsfactoren die worden gebruikt bij normafleiding.
Bij sterke synergetische interacties tussen stoffen is het echter mogelijk dat de risico voor bijen wordt onderschat omdat de generieke veiligheidsfactoren niet afdoende zijn. Het zal moeten worden onderzocht of er voor synergisme van deze stoffen aanvullende maatregelen nodig zijn in de risicobeoordeling of bij risicomanagement.

Op basis van openbare literatuur en de EFSA scientific contribution over bijen van 2012 blijkt dat neonicotinoïden niet de enige groep stoffen is waar synergisme optreedt. Er wordt op dit moment onderzocht in hoeverre andere werkzame stoffen ook synergisme vertonen. EFSA zal worden gevraagd om in samenwerking met Ctgb en andere kennisinstituten, een prioriteitenlijst van werkzame stoffen met synergistische interacties op te stellen.

In afwachting van een prioritering van synergistische interacties, opgesteld door EFSA of nationaal, heeft het College besloten nog geen versnelde herbeoordeling van de middelen op basis van thiacloprid en acetamiprid uit te voeren. Het zou als buitenproportioneel kunnen worden gezien indien neonicotinoïden worden herbeoordeeld als zij een lage prioritering hebben op deze lijst.

Wij verwachten in februari 2014 uitspraak te kunnen doen of een versnelde herbeoordeling van deze middelen noodzakelijk wordt geacht.

Met vriendelijke groet,

Appendix II Mode of action – predictor of synergistic mixture interactions?

This paper focuses on the possibility of predicting synergistic mixture interactions for ecological risk assessment. By exploring:

- The accuracy of reference models used to predict mixture effects.
- Methodologies to include the Mode of Action (MoA) to predict synergism.
- (Ecological) Risk Assessment frameworks that have attempted to include cumulative exposure in (E)RA.

Environmental fate of chemicals and other important biological availability aspects are not included or described.

Main Conclusions

The MoA of a chemical is often not the same for different organisms. In addition, interactions leading to synergistic mixture effects may take place in the toxicokinetic as well as in the toxicodynamics –phase (or both). However, reviews on pesticide mixture data revealed that most mixture effects are accurately described by the concept of concentration addition. Severe adverse synergism is rare.

Mixture effects should be included in ERA, but it is currently not feasible to include it in the ERA of individual plant protection products!

No validated methods are available to predict synergistic mixture interactions. However, although lacking robust data, the reference model CA seems to accurately describe most tested mixtures, and including the use of this model in the ERA is a very good start.

The current PPP regulation does not give a legal basis to include mixture toxicity in the current ERA of individual ppps.

Main knowledge gaps and complicating factors for the prediction of synergism

•Knowledge of the MoA of the individual components, including dose-response information is lacking
•Precise endpoints that in human toxicology often refer to a specific target organ cannot be used in ecotoxicology.
•MoA of chemicals is often not the same for different types of organisms and the knowledge about the MoA is usually poor.
•Ecotoxicological endpoint are relatively broad and are related to ecologically-relevant parameters (eg. massive mortality, reproduction, biomass) instead of a specific target organ.
•More informative data from single chemical toxicity tests should be provided (development of toxicity in time, dose-response-curve etc.) not only the endpoint under assessment. This may enable TK/TD modelling of the chemicals under assessment.
•No clear criteria are defined for 'similar' mode of action.
•Potential for synergism increases from the cellular level onwards, because interferences with uptake processes and metabolic steps may come into play.
•Different endpoints may show different mixture effects.
•Mixture interactions can take place in many different areas within an ecosystem (for instance prey-predator relationships)
•Mixture effects vary according to the relevant dose level, route of exposure, timing, life stage, endpoint and exposure duration of the biological target.

Recommendations to implement mixture toxicity in ERA

•Build a DATABASE with all known information on mixture effects, dose-response curves, time to effect, mode of action etc. and identify potential gaps in knowledge
•Identify and prioritise the compounds that occur together at relevant concentrations for different organisms (depending on the ecosystem/population).
•Determination of field relevant exposure levels, as interactions are often dose-dependent.
•Provide risk assessors with all available information from studies: Besides the ECx, NOELs, this requires information on measurements in time, dose-response curves etc.
•Develop a screening method for potential 'high' risk mixtures.
•Identify the MoA of chemicals for the endpoints under assessment.
•More information regarding association between groups of chemicals demonstrating similar or identical MoA (assessment groups)
•Predict ecological mixture effect according to the reference model concentration addition.

Introduction

In plant protection, different plant protection products (ppp) like insecticides, fungicides and herbicides are used to prevent, cure and exterminate diseases and pests that have severe adverse effects on crop yield and quality. These different ppp's can be applied simultaneously or alternatingly. Besides the target organisms, non-target organisms can be exposed to these ppp too. These non-target organisms may be potentially exposed to a plethora of different pesticides within one crop cycle. In addition, adjoining fields may also be treated with various (different) pesticides and active ingredients may be broken down over time into toxic metabolites. All these factors give rise to a complex mixture of different chemicals that may vary in space and time to which organisms can be exposed.

When an organisms is exposed to (a) chemical(s), toxicokinetics (TK) describes how much of the chemical is absorbed, distributed, metabolized and excreted (ADME) in the organism. Toxicodynamics (TD) describes how the (mixture) toxicity is caused once the biological target in the organism has been reached. It is often difficult to distinguish between TK and TD, especially when dealing with mixtures, since chemicals may interfere with each other in both phases.

Describing mixture toxicity

A necessary step in evaluating the risk of exposure to multiple known xenobiotics and, hence, mixture toxicity, is to make a prediction of the expected mixture effects. The concepts of Concentration Addition (CA)) and Independent Action (IA, also termed Response Addition) allow valid calculations of expected effects if the toxicity of the individual mixture components are known. CA assumes similar action of the chemicals in the mixture, while Independent Action takes dissimilar action as the starting point. In practice, this means that CA is used as the reference when testing chemicals with the same or similar modes of action, while Independent Action is the preferred reference model in case of chemicals with different modes of action.

For a given mixture, the observed effect can deviate from the predicted mixture effect (predicted by either IA or CA) and be more severe than predicted (synergism), or the effect of the mixture can be less than predicted (antagonism).

In practice, the applicability of these reference models is difficult, since no clear criteria are defined for 'similar mode of action'. Currently there is no convincing alternative approach to solve these difficulties. However, in practice, several authors have assessed the accuracy of CA to predict mixture toxicity for ppps: (cited by Van Gestel *et al.* 2011)."Deneer (2000) reviewed the usefulness of CA for describing combination effects of pesticides on aquatic organisms. (...) The review was based on experimental investigations of 202 mostly binary pesticide mixtures from 26 different studies, dating from 1972-1998. Results were reported for toxicity assays using fish, crustaceans, insects, molluscs and algae. For more than 90% of the studies, CA was found to predict mixture effect concentrations correctly within a factor of two, despite the fact that the assumption of a similar mode of action was violated by 85 of the mixtures under investigation. In a more recent review, Belden et al. (2007) evaluated 45 studies dealing with 303 pesticide mixture experiments. The authors quantified the difference between predicted and observed mixture effect concentrations. In 88% of the studies that could be evaluated using CA, the predicted mixture effect concentrations differed by no more than a factor of two from the observed effect concentrations, again irrespective of the involved mode of action of the mixture components."

Conclusion
It seems that predicting mixture effects according to the reference model of CA works reasonable well. Even if the underlying mechanisms might not be adequately represented by both models. In addition, in ecotoxicology, CA usually produced more conservative predictions when compared with IA. (In human RA IA is often used as the default reference model in toxicological mixture assessment).

Deviations from the reference models
In view of the aforementioned findings, the main challenge for mixture assessment is not so much to debate the average cases (for which the solution appears empirically right), but to identify and assess those mixtures that *significantly deviate* from the default models. Based on a review of available literature data, Warne (2003) concludes based on data from laboratory tests that approximately 10-15%, 70-80% and 10-15% of mixtures show antagonistic, additive and synergistic toxicity respectively. Warne (2003) also showed that with an increasing number of chemicals present in the mixture, the antagonistic and synergistic interactions seemed to be decreasing (the funnel hypothesis). Analyses by Ross (1996) and Ross and Warne (1997) indicated that 5% of the mixtures had toxicity that differed more than a factor of 2.5 from CA, and 1% of the mixtures had toxicity values that were different by more than a factor of 5.

The majority of the studies described by Deneer (2000) and Belden *et al.* (2007) have been carried out using organism-based assays. The following is taken from chapter 3 of van Gestel et al. (2011*): "The question arises to what extent the likelihood of agreement of observed mixture effects with additivity expectations varies when moving up or down the level of biological complexity, from the molecular to the population level or even the community level.*
Experimental evaluations of combination effects at the cellular or subcellular level from suspected endocrine disruptors or mycotoxins have demonstrated the usefulness of CA (Kortenkamp 2007; Speijers and Speijers 2004). However, on the grounds of theoretical considerations, this may not be surprising.

For biological responses at the molecular level, e.g. enzyme activities or receptor interactions, the dilution principle that is at the heart of CA can be readily interpreted in terms of molecular interactions. For this reason, other than concentration-additive effects may be difficult to envisage, and there is little scope for IA or substantial deviations from additivity suggesting synergism or antagonism. The likelihood of observing mixture effects accurately described by IA might increase as we move from the molecular to the cellular level. Cellular responses may be the result of interacting signalling pathways and diverse mechanisms and these effects might follow the IA principle. Similarly, the potential for synergisms or antagonisms increases from the cellular level onwards, because interferences with uptake processes and metabolic steps may come into play. Such phenomena are not accessible at the level of isolated enzymes or with biological responses very close to receptor activation."

In an opinion of SCHER (2012) it was concluded that interactions (including antagonism, potentiation, and synergism) usually occur at medium or high dose levels (relative to the lowest effect levels). At low exposure levels, they are either unlikely to occur or are toxicologically insignificant. EFSA (2008) concluded that significant toxic interactions between chemicals are 'much less likely to occur at doses below the effects level for individual compounds'.

The U.S. EPA suggested that low-dose regions of mixtures of chemicals should be associated with additivity, while interactions might occur in higher-dose regions. Hamm *et al.* (2005) applied a linear model to mixture effect data and estimated the location of the 'interaction threshold' boundary, for single chemicals the existence of effect thresholds has been extensively described. The interaction threshold might help determining whether departures from additivity in the mixture effects exist.

To complicate matters further, it has been recognized that different endpoints may result in different mixture effects. For instance, a mixture may show synergism when its effect on reproduction is analyzed, but CA for its effect on survival. Such a difference in interaction may hold mechanistic clues, but unless the mechanism is identified it is a complicating factor for including interactions in ERA where reproduction and mortality are considered the relevant endpoints.

The age of the exposed organism may also influence the severity of the adverse effects, as not all life stages are necessarily equally sensitive (or susceptible, for that matter). The exposure duration may also influence mixture effects, as it is widely known that effect concentrations are exposure duration-dependent (see e.g. Reynders et al. 2006). These observations indicate that time should be included in the mixture data analysis in order to make general statements about interactions.

Conclusion
So mixture effects (and hence mixture interactions) may occur in the TK and/or the TD phase. And these interactions may vary according to the relative dose levels, route(s), timing, life stage, endpoint assessed and duration of exposure, as well as the biological targets.

Detecting synergism
In the scientific literature, mixture interactions between ppp are well described. For instance, synergism between fungicides and insecticides are described by various authors (e.g. Pilling and Jepson, 1993; Colin and Belzunces, 1992; Iwasa et al. 2004). The ultimate goal in assessing mixtures would be to be able to predict mixture interactions without actually performing a mixture effect assessment study.

In the SCHER opinion (2012) a short list of considerations about a potential for toxicological significant synergistic effects is given:

- Can one or more components significantly enhance the uptake of other components?
- Can one or more components inhibit significantly the excretion/clearance of other components?
- Do one or more of the components exert toxic action via the formulation of an active metabolite(s) and might one or more of the components induce the drug metabolizing enzymes that may be involved in the formation of these active metabolite(s)?
- Can two or more components act on different enzymes in an important metabolic pathway?
- Can two or more components act on different elements of cellular protection mechanisms or cellular repair mechanisms?

In order to answer some of the consideration mentioned above, relevant knowledge on the mode/mechanism of action is necessary. As noted before CA and IA rely on an assumption of the MoA of the mixture constituents as well.

Mode/mechanism of action

Mode of action refers to the type of response produced in an exposed organism or to the critical steps or features of the mechanism required for production of the particular biological response. MoA should consider some aspects of the critical biochemical pathway and the resulting physiological and behavioural changes produced by alterations in that pathway by the toxic agent (Borgert *et al.* 2004).

A postulated MoA , then, is a biologically plausible sequence of 'key events' leading to an observed effect supported by robust experimental observations and mechanistic data (Meek 2009). In Appendix 1 an overview of possible synergistic interactions based on the individual MoA as indicated by Glavan (2013) is given.

Major differences exist between the amount of available information on MoA of chemicals for humans and other organisms. In pharmacology, specific biochemical interactions through which drug substances produce pharmacological effects are often known. In other fields methodologies to screen substances for their MoA have also been developed, such as toxicity pathways based on *in silico* and *in vitro* methodology that may become feasible to identify common modes of action. However, both SCHER (2012) and the report of the US national Academic's standing Committee on Use of Emerging Science of Environmental Health Decision concluded that 'many challenges remain to be addressed before the findings from high-throughput screens and *in silico* models may be considered sufficiently robust and informative'.

In ecological science, the need to understand the MoA of a chemical in order to accurately predict and understand effects is widely recognized. Unfortunately besides the difficulties that toxicology is facing, additional problems arise in ecotoxicology.

As stated in the SCHER opinion (2012) in ecotoxicology, "*knowledge of the toxicological MoA on all the different types of organisms that may be present in an ecosystem is largely incomplete. Even for chemicals developed with the objective of a specific action (pesticides!) the toxicological MoA is well known for target organisms, but not for the non-target organisms. Pesticides exert their effect on a particular physiological or metabolic function that usually, is not common to all living organisms present in a biological community.*

Therefore, for non-target organisms, taxonomically far from the target ones, the effect of the chemical is likely to be of the narcotic-type (baseline toxicity). (….) Therefore, the concept of 'common mode of action' may have a different meaning in ecotoxicology in comparison with human toxicology, and should be referred to broader end-points, such as reproduction impairment, population growth, mortality, etc."

Further, the MoA of a chemical might also be different depending on the concentration. McCarty and Mackay (1993) interpreted data from Hermens et al. and indicated that when chemicals are present in a mixture at concentrations below 0.3-0.02 times their threshold for a specific toxicity, their combined action does not occur as a consequence of the *specific mechanism* of that toxicity. In concentrations below their threshold for specific toxicity, organic chemicals are believed to merely contribute to an overall nonspecific narcotic effect.
However, despite these complications, the need to determine specific Mode(s) of action of chemicals is widely recognised. Based on existing knowledge, the construction of cumulative assessment groups of chemicals via a (presumed) 'similar' MoA has been issued (o.a. ECHA, OECD, ECHA, EPA) There is currently no general agreement on the best scientific approach for the grouping of chemicals, so it is most often done by expert judgement on a case-by-case basis (SCHER, 2012).

In addition, a common MoA can be insufficient for understanding interaction potential, because it fails to consider toxicokinetic interference (Lambert and Lipscomb, cited by Hertzberg *et al.* 2013).

Mixture toxicity and Risk Assessment
The great majority of (ecological) risk assessments ((E)RA) only focus on single chemicals. Even though it is acknowledged that humans and ecosystems are continually exposed to a very complex mixture of xenobiotics, no guidance is available on when the assessment of combination chemicals should be carried out.

For human RA, the European Commission Committees recognise that the 'no observed adverse effects' levels (NOAELs) derived experimentally do not always represent absolute zero-effect levels due to lack of statistical power. However, they conclude that conservative assumptions made when deriving safe levels for humans by using the application of uncertainty factors, render the possibility of mixture effects unlikely following exposure at said safe levels. This would be the case if the diversity of chemicals which make up the exposure act in strictly independent ways, according to the stochastic principles of the mixture concept of independent action (IA) (Bliss, 1939). Under these conditions, combined effect is not expected if all components in the mixture are presented at levels equivalent to zero effects. Multiple publications, however, assessed the presence of toxic effects of mixtures of compounds even though the individual stressors were present at concentrations below their No Observable Effect Concentration (NOEC) (e.g. Brian et al. 2007; Kortenkamp, 2008; Silva et al. 2002). Based on the assumptions of behind CA chemicals can contribute to the overall mixture effect in concentrations below their individual NOECs.

In the Netherlands, the presence of multiple active ingredients (a.i.s.) in one ppp formulation is included in the ecological risk assessment (ERA) by (assuming some sort of) CA of the individual a.i.s, based on only the endpoint of interest. In addition, formulation data for some endpoints are available. Data regarding the slope of the dose-response curve or the development of the toxicity in time of the individual a.i.s is not taken into account. As testing for mixture toxicity is not a data requirement, possible deviations from the mixture model CA may not be detected.

Synergistic mixture interactions from cumulative exposure may exceed the protection levels that are regarded safe by the ERA (for the multiple a.i.s assessed and for cumulative exposure in the environment). In addition, commonly used assessment factors (AF) in chemical registration/authorization processes cover a range of uncertainties, like laboratory to field extrapolations, but are not intended to account for mixture effects (Backhaus et al. 2013, Martin et al. 2013; Kortenkamp et al. 2009).

Institutes like EFSA, the U.S. EPA and ATSDR work on providing regulatory guidance for chemical authorisation bodies when conducting mixture risk assessments. Up till now, no final guidance is available for ecotoxiological risk assessment. However, for the cumulative assessment of risk for humans, multiple documents and opinions are available dealing with the question how to predict deviations from CA (and IA) based on the mode/mechanism of action (MoA) of the a.i.'s under investigation (ATSDR, 2001a, 2001b; U.S. EPA, 1986, 1988, 1999, 200a, 200b, 2001; EFSA 2008, 2009, 2013).

The US EPA has evolved one of the most elaborate regulatory frameworks for cumulative risk assessment (CRA). It starts with the identification of a common mechanism group where pesticides that induce a common toxic effect by a common mechanism of toxicity are grouped together. For US EPA a common mechanism of toxicity exists if the a.i. act the same way if the same toxic effect occurs in the same organ or tissue by essentially the same sequence of biochemical events. Common mechanism groups are then used to define common assessment groups. The US EPA has conducted CRA for five groups of pesticides: organ phosphorus compounds, N-methyl carbamates, s-trazines, chloroacetanilides and pyrethrins/pyretroids. If it is determined that chemicals have the same MoA (belong to the same assessment group), the exposure values of these chemicals will be aggregated in the RA calculation. However, this method cannot be used to determine potential synergisms of the mixture components. It merely determines the (concentration) risk bar for cumulative exposure of a group of similar acting chemicals. (Appendix 2 gives the method of constructing a cumulative assessment group taken from EFSA (2009)).

Assessing potential additive risks from PPPs

The potential additive risk from ppps used in one crop cycle to non-target organisms can be taken into account (based on the already calculated exposure concentrations and endpoints available). Data from environmental concentration measurements can give an indication of the exposure mixture of ppps in water, soil and other exposure media, and help to further prioritize which mixtures (in effect concentrations) are most often encountered.

That said, more information can and should be assessed from (standardized) toxicity tests. Development of toxicity over time, multiple endpoints, etc. could be recorded, shifting from default to a more detailed behavioural pattern for the chemical under investigation, as well as the test organism. This was illustrated in Figure 1 for human risk assessment by Meek (2009).

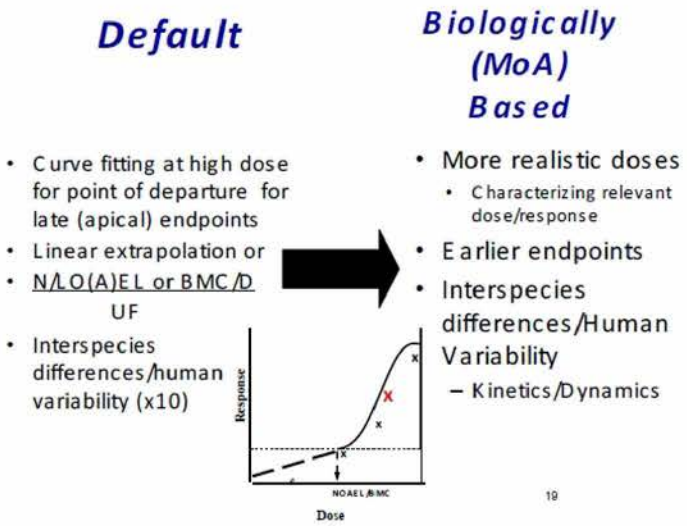

Figure 1. Moving from Default to More Mode of Action Based Approaches in Chemical Risk Assessment

Until sound methodologies are developed, the likelihood of synergistic interactions at actually relevant exposure levels has to be assessed on a case-by-case basis from mode of action information on the individual chemicals. But addressing only synergism in the risk assessment of individual ppps is insufficient if mixture toxicity itself is not fully addressed.

Main conclusions

The MoA of a chemical is often not the same for different organisms.

In addition, interactions leading to synergistic mixture effects may take place in the toxicokinetic as well as in the toxicodynamic –phase (or both) and depend on multiple factors. However, reviews on pesticide mixture data revealed that most mixture effects are accurately described by the concept of concentration addition. Severe adverse synergism is rare.

No methods are yet available to predict synergistic mixture interactions. However, although lacking robust data, the reference model CA seems to accurately describe most tested mixtures, including the use of this model in the ERA is a very good start.

Mixture effects should be considered to be included in ERA, but it is currently not feasible to include it in the ERA of individual plant protection products.

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Appendix II-I The proposed MoA leading to synergistic mixture effects in bees. Glavan et al. 2013 presented an overview of synergistic mixture interactions described for honeybees, together with the presumed MoA of the interaction. The summary is given in Table 1.

Table 1. List of synergisms of xenobiotics in honeybee Apis mellifera and the proposed mechanisms (taken from Glavan et al. 2013)

Xenobiotic	Xenobiotic (P450 inhibitor)	Reference
Mechanism of synergy: inhibition of P450 detoxifying enzymes		
pyrethroid insecticides	*classical P450-inhibitor*	
cyfluthrin	piperonyl butoxide	(Johnson et al. 2006)
permethrin	piperonyl butoxide	(Hagler et al. 1989)
lambda-cyhalothrin	piperonyl butoxide	(Johnson et al. 2006)
tau-fluvalinate	piperonyl butoxide	(Johnson et al. 2006; Johnson et al. 2013)
neonicotinoid insecticides	*classical P450-inhibitor*	
imidacloprid	piperonyl butoxide	(Iwasa et al., 2004, Johnson et al. 2012)
acetadimiprid	piperonyl butoxide	(Iwasa et al. 2004)
thiacloprid	piperonyl butoxide	(Iwasa et al. 2004)
carbamate insecticide	*classical P450-inhibitor*	
carbaryl	piperonyl butoxide	(Georghiou and Atkins Jr. 1964)
hive varroacides	*classical P450-inhibitor*	
tau-fluvalinate	piperonyl butoxide	(Johnson et al. 2009, Johnson et al. 2013)
coumaphos	piperonyl butoxide	(Johnson et al. 2009, Johnson et al. 2013)
fenpyroximate	piperonyl butoxide	(Johnson et al., 2013)
neonicotinoid insecticides	*EBI (ergosterol biosynthesis inhibitor) fungicides*	
acetamiprid	epoxiconazole, propiconazole, triadimefon, triflumizole, uniconazole-P	(Iwasa et al. 2004)
thiacloprid	prochloraz, propiconazole, tebuconazole, triflumizole	(Schmuck et al. 2003, Iwasa et al. 2004)
imidacloprid	propiconazole, triflumizole	(Iwasa et al. 2004)
pyrethroid insecticides	*EBI (ergosterol biosynthesis inhibitor) fungicides*	
deltamethrin	difenoconazole+carbendazim, prochloraz, prochloraz+ difenoconazole 850	(Belzunces and Colin 1993, Colin and Belzunces 1992, Papaefthimiou and Theophilidis 2001, Vandame and Belzunces1998b, Vandame and Belzunces 1998a)
lambda-cyhalothrin	difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole, thiophanate-methyl	(Thompson and Wilkins 2003)
alphacypermethrin	difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole	(Thompson and Wilkins 2003)
hive varroacides	*EBI (ergosterol biosynthesis inhibitor) fungicides*	
coumaphos	prochloraz	(Johnson et al. 2013)
flumethrin	carbendazim, difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole, thiophanate-methyl	(Thompson and Wilkins 2003)

Xenobiotic	Xenobiotic (P450 inhibitor)	Reference
tau-fluvalinate	carbendazim, difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole, thiophanate-methyl, myclobutanil, metconazole, fenbuconazole,	(Thompson and Wilkins 2003, Johnson et al. 2013)
fenpyroximate	prochloraz	(Johnson et al. 2013)
hive varroacides	*hive varroacides*	
coumaphos	tau-fluvalinate	(Johnson et al. 2009, 2013)
thymol	tau-fluvalinate, coumaphos	(Johnson et al. 2013)
amitraz	tau-fluvalinate, coumaphos, fenpyroximate	(Johnson et al. 2013)
fenpyroximate	tau-fluvalinate, coumaphos	(Johnson et al. 2013)
Mechanism of synergy: increased oxidative stress		
hive varroacides	*Fungicides (mitochondrial inhibitors)*	
tau-fluvalinate	pyraclostrobin, boscalid	(Johnson et al. 2013)
fenpyroximate	pyraclostrobin	(Johnson et al. 2013)
Unknown mechanism of synergy		
oxalic acid	tau-fluvalinate, fenpyroximate, amitraz, thymol	(Johnson et al. 2013)
herbicide atrazine	carbamate insecticides (carbaryl, carbofuran)	(Sonnet et al. 1978)
thio and dithiophosphoric ester pesticides – ethyl parathion, dimethoate, dialifos	coumaphos varroacide	(Lienau 1990)
thiacloprid (neonicotionoid)	fungicides cyprodinil, tolyfluanid	(Schmuck et al. 2003)
alphacypermethrin, lambda-cyhalothrin	fungicide chlorothalonil fungicide chlorothalonil	(Thompson and Wilkins 2003)

Especially for the known P450 inhibiting substances, the presumed synergistic MoA is believed to take place via inhibition of the detoxifying enzymes. However, some of the same active substances are also involved in completely unidentified synergistic interactions (even though the MoA of the individual compounds for the target organism is more or less known).

Appendix III Review of the available mixture studies in scientific literature assessing mixture effects on honey bees and the presence in bee-products.

Bees are important pollinators of both managed crops and wild flora. Honey bees can forage over a large area, with a mean radius of 5 km from their hive giving a potential foraging surface of over 7500 ha. This increases the chance of being exposed to different stressors, eg. xenobiotics like pesticides. Given the complicated structure of a honey bee colony, this may not only pose a risk for the individual bee, but for the whole hive. Polluted pollen, nectar etc can be taken back to the hive, where a combination of all these products from different sources can expose the bees to a mixture of a wide range of xenobiotics.
This review focuses mainly on known effects of the active substances (a.s.) in pesticides, tested in mixtures on honey bees and the presence of the a.s. in bee products, like pollen, honey, comb wax, bee bread and dead honey bees.

EFSA (2012;10(5):2668): "Honey bees and Hymenoptera are known to have a specific metabolic profile with the lowest number of copies of detoxification enzymes within the insect kingdom. A number of studies have shown synergistic effects of pesticides and active substances applied in hives as medical treatments against *Varroa* mites in honey bees, for which toxicokinetic interactions were most commonly involved.
There is also a growing body of evidence of interaction between honey bee disease (fungi, bacteria and viruses) and pesticides. Currently, full dose responses for synergistic effects between potential inhibitors and different classes of pesticides are rarely available for either lethal effects or sub-lethal effects in bees so that predictions of the magnitude of these interactions at realistic exposure levels cannot be performed. However, there is evidence that where realistic exposure levels have been investigated, deviations from concentration addition, such as synergy, is rarely more than a factor of 2 to 3. Such deviations have been observed for mixtures containing small numbers of chemicals and decreases as the complexity of the mixture increases.

In some cases, a response in between concentration addition and response addition has been noticed. Mixture effects cannot be ruled out, even when all compounds in a mixture with different MOA are present at their individual NOECs.
Synergistic or antagonistic effects are more uncommon. Indeed, deviations from the predictive concentration addition model, indicative of synergisms or antagonisms, are comparatively rarer, relatively small and largely confined to mixtures with only a few compounds.
In principle, the toxicology of mixtures involves two potential types of interactions: toxicokinetic and toxicodynamic interactions."

In the Appendix multiple tables are given, Table 2.12A, 2.13, 2.15, 2.16, and 1 give an overview of the known studies that examined mixture effects of binary pesticide and or varroacides mixtures on honey bees. On basis of the result, only pesticides and varroacides that were found to be involved in mixture interactions (antagonistic or synergistic) greater than 3-fold (on basis of the endpoint assessed for the single chemical) or effects > 10% were further examined. For these chemicals there presence in pollen, honey, comb wax, bee bread and/or dead honeybees was examined (on basis of the results given in tables G1, 7, G6, 1.10 and 7 in the Appendix). Result are given in the following table 1. The last column shows if the particular a.s. is accepted in the Netherlands.

Table 1. Summary of all the tables presented in the Appendix. Only active substances that were involved in mixture interactions >3 fold or > 10% are presented. The + or – signs indicates if the active substance is found in pollen, bee bread, honey, comb wax, and or dead honey bees. The last column indicates if the a.s. is accepted in the Netherlands.

Pesticide	Pollen	Bee bread	Honey	Comb wax	Honey bees	Accepted in NL
2,4 D	-	-	-	-	-	N
Acetamiprid	+[2]	+	+[3]	+[1]	-	Y
Alphacypermethrin	-	-	-	-	-	N
Aldrin	-	-	+	-	-	N
Coumaphos	+	+	+	+	+	N
Carbaryl	+	+	+	-	+	N
Carbendazim	+	-	+	-	+	Y
Cyfluthrin	+	-	-	+	+	Y
Cyprodinil	-	+	-	-	-	Y
Deltamethrin	+	-	+	+	+	Y
Dieldrin	+	-	-	-	+	N
Difenoconazole	+	+	+	-	-	Y
Flumethrin	+	-	+	+	-	N
Fluvalinate	+	-	+	+	+	N
Flutriafol	-	-	-	-	-	N
Flusilazole	+	+	+	+	+	N
Imazalil	-	-	+	-	+	Y
Iprodione	+	+	+	-	+	Y
Lambda-cyhalothrin	-	+	+	-	+	Y
Mancozeb	-	-	-	-	-	Y
Malathion	+	-	+	+	+	N
Methyl parathion	-	+	-	-	-	N
Monuron oral	-	-	-	-	-	N
Myclobutanil	+	+	+	-	+	N
PBO		-				Y
Permethrin	-	-	-	-	-	Y
Phenobabital	-	-	-	-	-	N
Prochloraz	-	-	+	-	-	Y
Propiconazole	-	-	-	-	+	Y
Simazine	-	-	+	-	-	N
Tau-fluvalinate	+	+	+	+	+	N
Tebuconazole	+	+	+	-	+	Y
Thiacloprid	+[2]	+	-	-	-	Y
Thiophanate methyl	+	-	+	-	+	N
Tolyfluanid (phenylsylfamide)	-	+	-	-	-	Y
Triademefon	-	-	-	-	-	N
Triflumizole	-	-	-	-	-	Y
Uniconazole-P	-	-	-	-	-	N

1. (Yáñez, Bernal et al. 2013) Spain
2. (Pohorecka, Skubida et al. 2012) Poland
3. (Mullin et al. 2010) North America

As can be seen in the Tables on mixture effects in the Appendix, it is difficult to give a general conclusion on which active substances (a.s.) are likely to be involved in a mixture interaction.
However, a few trends are observed:

- Insecticides tend to interact with classic synergists (PBO)
- Neonicotinoids tend to interact with EBI fungicides
- Lambda-cyhalothrin often seems to be involved in mixture interactions.

Results for only a few semi-field studies are known (Table 2.15, Appendix) with only a few a.s.. In these studies no mixture interactions were found. However, most of the combinations tested 'positive' in the laboratory are not tested in the field.

As shown in table 2.16 (Appendix), mixture interactions were also found between pesticides and varroacides. However, even fewer studies examined the effects between these groups of xenobiotics than for pesticide mixtures.

With regards to the residue data presented in the Appendix, most studies only measured the residue of one particular a.s., not the presence of all residues, which may greatly underestimate the residues found in pollen, honey, bee bread, comb wax and dead honey bees. Secondly, none of the residue measurements were performed in the Netherlands.

With regards to the residues measured in pollen, honey, bee bread and comb wax, these residues only reflect the pesticides and the exposure concentrations that still enabled the honey bee to return to the hive. The dead honey bees examined at least make it back to the hive. No records are known of residues measured in dead bees found in the field.

This greatly complicates the estimation of the potential risk for honey bees from tankmixes. Also the absence of residues in the hive does not necessarily indicate that honey bees were not exposed to a high risk. A.s. can have a very low DT50, and be present in concentrations < LOQ after a few days.

Concluding, in the laboratory mixture interactions are found that increase or decrease the endpoints of the single a.s. more than 3 fold or over 10%. Only a few semi-field studies are known that examined the effects of a very limited amount of a.s. in a more 'real life'situation. No mixture interactions were detected. However, the amount of combinations and studies are so limited, that mixture effects in the field cannot be excluded on the basis of these results.

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Appendix III-I Supporting information for the review.

Table 2.12A and table 2.13 give an overview of the known studies available that assessed the possibility of mixture interactions between pesticides.

only pesticides and varroacides that were found to be involved in mixture interactions (antagonistic or synergistic) greater than 3-fold (on basis of the endpoint assessed for the single chemical) or effects > 10% were further examined.
Interactions (antagonistic or synergistic) that are > 3 fold, or have a >10% are marked yellow.

Table 2.12A: Synergism of three pyrethroid insecticides administered 1 hr after treatment with acetone control or detoxification enzyme inhibitors (DEM, a glutathione-S-transferase inhibitor; DEF, a carboxylesterase inhibitor; or PBO, a cytochrome P450 monooxygenase inhibitor) (Johnson et al., 2006)

Insecticide	Inhibitor	n	LD50 ng/bee (95% CI)	Synergism		
				X_2	DF	Ratio LD50
Cyfluthrin	None	440	62.0(67.7-74.5)	2.9	4	-
	DEF	280	29.0(20.5-51.5)	9.5	3	2.3*
	PBO	380	2.24(1.48-4.18)	13.8	4	30*
Lambda-cyhalothrin	None	360	102(73.0-133)	6.0	3	-
	DEM	260	38.0(27.5-50.7)	3.2	3	2.7*
	PBO	360	1.28(1.12-1.46)	2.0	3	80*
Tau-fluvalinate	None	820	9450(7480-12000)	11.1	5	-
	DEM	620	8260 (7570-9030)	2.2	4	1.1ns
	DEF	480	1960(830-4170)	23.8	4	4.8*
	PBO	880	9.64(6.61-14.9)	11.6	4	980*

*Statistically significant, X_2: X_2 value; DF: degrees of freedom, Ratio LD50: ratio of LD50 control single compounds versus mixture.

Table 2.12A is taken from Thompson (2012).

Table 2.13 Summary of studies undertaken in the laboratory with honeybees and pesticide mixtures

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
Carbamate insecticides							
+ Classical synergists							
Sevin (carbaryl) contact	PBO ratio 1:5 contact	Mixture	Inc mortality from 38 to 97% mortality at 16C, 8 to 97% at 27C and 8 to 87% at 32C	Apis mellifera	Not stated	Active ingredient, 20/replicate, 4 replicates, 16, 27, 32 C, 60%RH control treated with acetone mortality 4, 8, 16, 24hrs, < 5% mortality in controls	(Georghiou and Atkins Jr, 1964)
+ herbicides							
Carbaryl 1 ,3, 5 ppm	Atrazine 100ppm oral	mixture	mortality 1.0-1.1 fold	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial random variable	(Sonnet et al., 1978)
Carbofuran 0.3, 0.5, 1 ppm	Atrazine 100ppm oral	mixture	mortality 0.2-1.0 fold	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial random variable	(Sonnet et al., 1978)
Neonicotinoids							
+ Classical synergists							
Acetamiprid (LD50 0.007 mg/bee) contact	PBO 0.010 mg/bee contact	Synergist 1hr prior	Inc LD50 6.0 fold	Apis mellifera	No treatment prior to study	Active ingredients, 30 insects per replicate, 5-7 doses, 2-3 replicates, 27±1°C, 50% RH, 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality, Statistics: Abbotts correction, probit analysis, Students t-test for means	(Iwasa et al., 2004a)
Imidacloprid (LD50 0.0000179 mg/bee) contact	PBO 0.010 mg/bee contact	Synergist 1hr prior	Inc LD50 1.7 fold	Apis mellifera	No treatment prior to study	Active ingredients, 30 insects per replicate, 5-7 doses, 2-3 replicates, 27±1°C, 50% RH, 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality Statistics: Abbotts correction, probit analysis, Students t-test for means	(Iwasa et al., 2004a)
Thiacloprid (LD50 0.0146 mg/bee) contact	PBO 0.010 mg/bee contact	Synergist 1hr prior	Inc LD50 154 fold	Apis mellifera	No treatment prior to study	Active ingredients, 30 insects per replicate, 5-7 doses, 2-3 replicates, 27±1°C, 50% RH, 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality Statistics: Abbotts correction, probit analysis, Students t-test for means	(Iwasa et al., 2004a)
Acetamiprid (LD50 0.007 mg/bee) contact	DEF 0.010 mg/bee contact	Synergist 1hr prior	Inc LD50 3.0 fold	Apis mellifera	No treatment prior to study	Active ingredients, 30 insects per replicate, 5-7 doses, 2-3 replicates, 27±1°C, 50% RH, 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality Statistics: Abbotts correction, probit analysis, Students t-test for means	(Iwasa et al., 2004a)
Acetamiprid (LD50 0.007 mg/bee) contact	DEM 0.010 mg/bee contact	Synergist 1hr prior	No effect on LD50	Apis mellifera	No treatment prior to study	Active ingredients, 30 insects per replicate, 5-7 doses, 2-3 replicates, 27±1°C, 50% RH, 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality Statistics: Abbotts correction, probit analysis, Students t-test for means	(Iwasa et al., 2004a)
+ EBI fungicides							
Acetamiprid (LD50	Epoxiconazole	Synergist	Inc LD50 14	Apis	No treatment	Active ingredients, 30 insects per	(Iwasa et al., 2004a)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
0.007 mg/bee) contact	0.010 mg/bee contact	1hr prior	fold	mellifera	prior to study	replicate, 5-7 doses, 2-3 replicates, 27±1°C, 50% RH, 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality	
Thiacloprid 0.001, 0.010 mg/bee contact	Prochloraz 0.001, 0.010 mg/bee contact	mixture	0% mortality at 0.001mg/bee; inc mortality from 10 to 87% at 0.01 mg/bee,	A mellifera carnica	no antibiotics or varrocides within 4 weeks	Formulations 25±2 °C, 50-60% RH, 24h dark, 3 replicates of 10 bees per dose level, mortality and behaviour (discoordinated movements, staggering, apathy) up to 96hrs, 0% control mortality	(Schmuck et al., 2003b)
Acetamiprid (LD50 0.007 mg/bee) contact	Propiconazole 0.010 mg/bee contact	Synergist 1hr prior	Inc LD50 105 fold	Apis mellifera	No treatment prior to study	Active ingredients, 30 insects per replicate, 5-7 doses, 2-3 replicates, 27±1°C, 50% RH, 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality Statistics: Abbotts correction, probit analysis, Students t-test for means	(Iwasa et al., 2004a)
Imidacloprid (LD50 0.0000179 mg/bee) contact	Propiconazole 0.010 mg/bee contact	Synergist 1hr prior	Inc LD50 1.5 fold	Apis mellifera	No treatment prior to study	Active ingredients, 30 insects per replicate, 5-7 doses, 2-3 replicates, 27±1°C, 50% RH, 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality Statistics: Abbotts correction, probit analysis, Students t-test for means	(Iwasa et al., 2004a)
Thiacloprid (LD50 0.0146 mg/bee) contact	Propiconazole 0.010 mg/bee contact	Synergist 1hr prior	Inc LD50 559 fold	Apis mellifera	No treatment prior to study	Active ingredients, 30 insects per replicate, 5-7 doses, 2-3 replicates, 27±1°C, 50% RH, 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality Statistics: Abbotts correction, probit	(Iwasa et al., 2004a)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
						analysis, Students t-test for means	
Thiacloprid 0.002 mg/bee contact	Tebuconazole 0.003 mg/bee contact	mixture	Inc mortality from 3 to 70%	A mellifera carnica	no antibiotics or varrocides within 4 weeks	Formulations 25±2 °C, 50-60% RH, 24hr dark, 3 replicates of 10 bees per dose level, mortality and behaviour (discoordinated movements, staggering, apathy) up to 96hrs, 0% control mortality	(Schmuck et al., 2003b)
Acetamiprid (LD50 0.007 mg/bee) contact	Triadimefon 0.010 mg/bee contact	Synergist 1hr prior	Inc LD50 84 fold	Apis mellifera	No treatment prior to study	Active ingredients, 30 insects per replicate, 5-7 doses, 2-3 replicates, 27±1°C, 50% RH, 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality Statistics: Abbotts correction, probit analysis, Students t-test for means	(Iwasa et al., 2004a)
Acetamiprid (LD50 0.007 mg/bee) contact	Triflumizole 0.010 mg/bee contact	Synergist 1hr prior	Inc LD50 244 fold	Apis mellifera	No treatment prior to study	Active ingredients, 30 insects per replicate, 5-7 doses, 2-3 replicates, 27±1°C, 50% RH, 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality Statistics: Abbotts correction, probit analysis, Students t-test for means	(Iwasa et al., 2004a)
Imidacloprid (LD50 0.0000179 mg/bee) contact	Triflumizole 0.010 mg/bee contact	Synergist 1hr prior	Inc LD50 1.9 fold	Apis mellifera	No treatment prior to study	Active ingredients, 30 insects per replicate, 5-7 doses, 2-3 replicates, 27±1°C, 50% RH, 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality Statistics: Abbotts correction, probit analysis, Students t-test for means	(Iwasa et al., 2004a)
Thiacloprid (LD50	Triflumizole	Synergist	Inc LD50	Apis	No treatment	Active ingredients, 30 insects per	(Iwasa et al., 2004a)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
0.0146 mg/bee) contact	0.010 mg/bee contact	1hr prior	1141 fold	mellifera	prior to study	replicate, 5-7 doses, 2-3 replicates, 27±1℃, 50% RH, 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality Statistics: Abbotts correction, probit analysis, Students t-test for means	
Acetamiprid (LD50 0.007 mg/bee) contact	Uniconazole-P 0.010 mg/bee contact	Synergist 1hr prior	Inc LD50 6.3 fold	Apis mellifera	No treatment prior to study	Active ingredients, 30 insects per replicate, 5-7 doses, 2-3 replicates, 27±1℃, 50% RH. 14:10 light :dark, mortality (incl knocked down) at 24hrs, 3.7% control mortality Statistics: Abbotts correction, probit analysis, Students t-test for means	(Iwasa et al., 2004a)
+ Other fungicides							
Thiacloprid 0.002 mg/bee contact	Cyprodinil (Anilino-pyrimidine) 0.008 mg/bee contact	mixture	Inc mortality from 3 to 20%	A mellifera carnica	no antibiotics or varrocides within 4 weeks	Formulations 25±2 °C, 50-60% RH, 24hr dark, 3 replicates of 10 bees per dose level, mortality and behaviour (discoordinated movements, staggering, apathy) up to 96hrs, 0% control mortality	(Schmuck et al., 2003b)
Thiacloprid 0.002 mg/bee contact	Azoxystrobin (methoxyacrylate strobilurin) 0.003 mg/bee contact	mixture	No effect on mortality	A mellifera carnica	no antibiotics or varrocides within 4 weeks	Formulations 25±2 °C, 50-60% RH, 24hr dark, 3 replicates of 10 bees per dose level, mortality and behaviour (discoordinated movements, staggering, apathy) up to 96hrs, 0% control mortality	(Schmuck et al., 2003b)
Thiacloprid 0.002 mg/bee contact	Tolyfluanid (phenylsulfamide) 0.011 mg/bee contact	mixture	Inc mortality from 3 to 13%	A mellifera carnica	no antibiotics or varrocides within 4 weeks	Formulations 25±2 °C, 50-60% RH, 24hr dark, 3 replicates of 10 bees per dose level, mortality and behaviour (uncoordinated movements, staggering, apathy) up to 96hrs, 0%	(Schmuck et al., 2003b)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
						control mortality	
Thiacloprid 0.002 mg/bee contact	Mancozeb (dithiocarbamate) 0.008 mg/bee contact	mixture	No effect on mortality	A mellifera carnica	no antibiotics or varrocides within 4 weeks	Formulations 25±2 °C, 50-60% RH 24hr dark , 3 replicates of 10 bees per dose level, mortality and behaviour (discoordinated movements, staggering, apathy) up to 96hrs, 0% control mortality	(Schmuck et al., 2003b)
Phenylpyrazole insecticides + EBI fungicides							
Fipronil contact (LD50 0.005546 µg/bee) and oral (LD50 0.1015µg/bee)	Trichlorfon contact (LD50 4.065 µg/bee) and oral (LD50 13.89 mg/bee)	mixture	Additive toxicity	Apis mellifera (Italian)	Not stated	LD50, 48hr mortality	(Cang et al., 2008) (abstract only available)
Organochlorine insecticides + classic synergists							
aldrin	Phenobarbital 5 mg/g candy oral	3 days prior	Dec LD50 from 60.5 to 38.5 ng/bee	Apis mellifera	Not stated	Active ingredients, 3 day old bees, 20 bees/replicate 32-34C, 24h mortality, Probit analysis	(Johnson et al., 2012)
dieldrin	Phenobarbital 5 mg/g candy oral	3 days prior	Dec LD50 from 37.2 to 20.7 ng/bee	Apis mellifera	Not stated	Active ingredients, 3 day old bees, 20 bees/replicate 32-34C, 24h mortality, Probit analysis	(Johnson et al., 2012)
Organophosphorus insecticides							
+ herbicides							
Malathion 3ppm oral	2,4D oral	mixture	dec mortality from 61 to 24%	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial random variable	(Sonnet et al., 1978)
Methyl parathion 1ppm oral	2,4D oral	mixture	dec mortality from 50 to 28%	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial	(Sonnet et al., 1978)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
						random variable	
Diazinon 3, 5 ppm	Atrazine 100ppm oral	mixture	dec mortality	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial random variable	(Sonnet et al., 1978)
Malathion 3, 5 pmm	Atrazine 100ppm oral	mixture	Dec mortality	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial random variable	(Sonnet et al., 1978)
Methyl parathion 1, 5 ppm	Atrazine 100ppm oral	mixture	Dec mortality	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial random variable	(Sonnet et al., 1978)
Mevinphos 0.3, 0.5 ppm	Atrazine 100ppm oral	mixture	dec mortality	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial random variable	(Sonnet et al., 1978)
Monocrotophos 0.5, 1 ppm	Atrazine 100ppm oral	mixture	Dec mortality	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial random variable	(Sonnet et al., 1978)
Parathion 7, 10 ppm	Atrazine 100ppm oral	mixture	Dec mortality	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial random variable	(Sonnet et al., 1978)

3

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
Malathion 3ppm oral	Monuron oral	mixture	dec mortality from 61 to 28%	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial random variable	(Sonnet et al., 1978)
Methyl parathion 1ppm oral	Monuron oral	mixture	dec mortality from 50-to 27%	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial random variable	(Sonnet et al., 1978)
Malathion 3ppm oral	Simazine oral	mixture	dec mortality 61-7%	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial random variable	(Sonnet et al., 1978)
Methyl parathion 1ppm oral	Simazine oral	mixture	dec mortality from 50-to 27%	Apis mellifera	Not stated	Commercial grade (?) 25°C, 40-45% RH, amount consumed not measured, 4 trials 30-60bees/trial control mortality not stated, statistics binomial random variable	(Sonnet et al., 1978)
+ organophosphorus insecticide							
Thio and dithiophosphoric ester pesticides – ethyl parathion, dimethoate, dialifos oral	Coumaphos varroacide	Pretreatment in artificial swarm four days prior	Inc toxicity	A mellifera carnica	Pretreatment with varroacide	Artificial swarm 34C, 70% RH 48hr mortality	(Lienau, 1990) (abstract only available)
Pyrethroids							
Lambda-cyhalothrin dose response LD50 contact	PBO contact (1:10 pyrethroid:PBO)	mixture	Dec LD50 from 0.15 ug ai/bee to 0.077 ug ai/bee (2.0	Apis mellifera	Not stated	Formulations, 25C, 75%RH mortality 24hr	(Pilling, 1992)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
			fold)				
Permethrin 0-75 ug/ml	PBO ratio 1:4 and 1:9 (pyrethroid:PBO)	Mixture	5.4 fold inc toxicity 1:4 9 fold increase toxicity 1:9	Apis mellifera	Not stated	Active ingredient, 24 C, 30% RH dark, filter paper exposure, 10 bees per dose, 6 doses, replicated 7 times,48hr mortality Probit analysis	(Hagler et al., 1989)
Lambda-cyhalothrin	Phenobarbital 5 mg/g candy oral	3 days prior	Dec LD50 from 47.5 to 16.9 ng/bee	Apis mellifera	Not stated	Active ingredients, 3 day old bees, 20 bees/replicate 32-34C, 24h mortality, Probit analysis	(Johnson et al., 2012)
+ EBI fungicides							
Alphacypermethrin	Carbendazim (ratio related to application rate) 1:66	mixture	No change in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003)(Defra PN0945)
Alphacypermethrin	Carbendazim (ratio related to application rate) 1:66	mixture	No change in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients,, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003)(Defra PN0945)
Alphacypermethrin	Carbendazim (ratio related to application rate) 1:33	mixture	1.6 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Folkard-Ward, 2001)
Lambda-cyhalothrin	Carbendazim (ratio related to application rate) 1:50	mixture	1.3 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t	(Thompson and Folkard-Ward, 2001)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
					weeks of start of study	test vs additive toxicity	
Lambda-cyhalothrin	Carbendazim (ratio related to application rate) 1:232	mixture	2.6 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003)(Defra PN0945)
Lambda-cyhalothrin	Carbendazim (ratio related to application rate) 1:232	mixture	1.3 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003)(Defra PN0945)
Alphacypermethrin	Difenconazole (ratio related to application rate) 1:9.4	mixture	1.2 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003)(Defra PN0945)
Alphacypermethrin	Difenconazole (ratio related to application rate) 1:9.4	mixture	1.8 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients,, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003)(Defra PN0945)
Deltamethrin 0.75 g ai/ha	Difenoconazole +carbendazim 125-250 g/ha (1:167-333)	mixture	Increased mortality	Apis mellifera	Not stated	Formulations sprayed in Potter Tower, 28±1°C, 50-70%RH, 50 /replicate, 12 replicates, 24-48hrs mortality,	(Belzunces and Colin, 1993) (Colin and Belzunces, 1992a)
Deltamethrin 0.75 g	Difenoconazole	sequential	No effect	Apis	Not stated	Formulations sprayed in Potter Tower,	(Belzunces and Colin,

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
ai/ha	+carbendazim 125-250 g/ha (1:167-333)			mellifera		28±1°C, 50-70%RH, 50 /replicate, 12 replicates, 24-96hrs mortality,	1993) (Colin and Belzunces, 1992a)
Lambda-cyhalothrin	Difenconazole (ratio related to application rate) 1:33	mixture	2.7 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003)(Defra PN0945)
Lambda-cyhalothrin	Difenconazole (ratio related to application rate) 1:33	mixture	No change in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003)(Defra PN0945)
Alphacypermethrin	Flusilazole (ratio related to application rate) 1:15	mixture	No change in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003)(Defra PN0945)
Alphacypermethrin	Flusilazole (ratio related to application rate) 1:15	mixture	2.5 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003)(Defra PN0945)
Lambda-cyhalothrin	Flusilazole (ratio related to application rate) 1:53	mixture	2.2 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t	(Thompson and Wilkins, 2003)(Defra PN0945)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
					weeks of start of study	test vs additive toxicity	
Lambda-cyhalothrin	Flusilazole (ratio related to application rate) 1:53	mixture	2.5 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003)(Defra PN0945)
Lambda-cyhalothrin dose response LD50 contact	Flutriafol (SC) ratio related to application rate (1:15)	mixture	Dec LD50 from 0.068 ug ai/bee to 0.0205 ug ai/bee (3.3 fold)	Apis mellifera	Not stated	Formulations, 25C, 75%RH, 10 bees per replicate, 3 replicates per dose, 6 doses, mortality 24hr	(Pilling and Jepson, 1993)
Lambda-cyhalothrin dose response LD50 contact	Flutriafol ratio related to application rate contact (1:16)	mixture	Dec LD50 from 0.15 ug ai/bee to 0.026 ug/bee (5.8 fold)	Apis mellifera	Not stated	Formulations, 25C, 75%RH mortality 24hr	(Pilling, 1992)
Lambda-cyhalothrin dose response LD50 contact	Imazalil (EC) ratio related to application rate (1:13.3)	mixture	Dec LD50 from 0.068 ug ai/bee to 0.0095 ug ai/bee (7.16 fold)	Apis mellifera	Not stated	Formulations, 25C, 75%RH, 10 bees per replicate, 3 replicates per dose, 6 doses, mortality 24hr	(Pilling and Jepson, 1993)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
Alphacypermethrin	Iprodione (ratio related to application rate) 1:50	mixture	6 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Folkard-Ward, 2001)
Lambda-cyhalothrin	Iprodione (ratio related to application rate) 1:75	mixture	1.5 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Folkard-Ward, 2001)
Deltamethrin 0.75 g ai/ha	Iprodione + carbendazim 525-262.5 g/ha	mixture	No effect	Apis mellifera	Not stated	Formulations sprayed in Potter Tower, 28±1°C, 50-70%RH, 50 /replicate, 12 replicates, 24-48hrs mortality,	(Belzunces and Colin, 1993)
Lambda-cyhalothrin dose response LD50 contact	Myclobutanil (SC) ratio related to application rate (1:12)	mixture	Dec LD50 from 0.068 ug ai/bee to 0.0089 ug ai/bee (7.64 fold)	Apis mellifera	Not stated	Formulations, 25C, 75%RH, 10 bees per replicate, 3 replicates per dose, 6 doses, mortality 24hr	(Pilling and Jepson, 1993)
Lambda-cyhalothrin dose response LD50 contact	Myclobutanil (WP) ratio related to application rate (1:12)	mixture	Dec LD50 from 0.068 ug ai/bee to 0.0048 ug ai/bee (14.1 fold)	Apis mellifera	Not stated	Formulations, 25C, 75%RH, 10 bees per replicate, 3 replicates per dose, 6 doses, mortality 24hr	(Pilling and Jepson, 1993)
Lambda-cyhalothrin	Penconazole (EC)	mixture	Dec LD50	Apis	Not stated	Formulations, 25C, 75%RH, 10 bees	(Pilling and Jepson, 1993)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
dose response LD50 contact	ratio related to application rate (1:6.6)		from 0.068 ug ai/bee to 0.0154 ug ai/bee (4.42 fold)	mellifera		per replicate, 3 replicates per dose, 6 doses, mortality 24hr	
Alphacypermethrin	Prochloraz (ratio related to application rate) 1:37	mixture	2.2 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Alphacypermethrin	Prochloraz (ratio related to application rate) 1:37	mixture	13 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Deltamethrin	Prochloraz	mixture	Isolated heart 100 fold inc cardiotoxicity of deltamethrin, 10 fold inc cardiotoxicity prochloraz Suggested joint action at target site in heart – gap junctional intercellular communicatio n	A mellifera macedonic a	Not stated	Active ingredients No temp info N=6 3 concentrations deltamethrin and prochloraz, six studies ANOVA and Dunnetts test	(Papaefthimiou and Theophilidis, 2001)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
Deltamethrin 0.125 g ai/ha	Prochloraz 25 g ai/ha (1:200)	Sequential separated by 0.8 days	Inc mortality from 0 to 23.8-27.5% at 50h (depending on order of applications)	Apis mellifera	Not stated	Active ingredients, sprayed in Potter-Tower, 24±1°C, 50-70%RH, 50/replicate, 3 replicates per dose 4 doses, ANOVA	(Belzunces and Colin, 1993; Colin and Belzunces, 1992b)
Deltamethrin 125 mg ai/ha	Prochloraz 25 g ai/ha (1:200)	Mixture potter tower	Inc mortality from 0 to 67.5% at 24 hrs, 74.1% at 50h	Apis mellifera	Not stated	Active ingredients, sprayed in Potter-Tower, 24±1°C, 50-70%RH, 50/replicate, 3 replicates per dose 4 doses, ANOVA	(Belzunces and Colin, 1993; Colin and Belzunces, 1992b)
Deltamethrin 125 mg/ha	Prochloraz 25 g/ha (1:200)	Mixture potter tower	63 ±5% mortality	Summer A mellifera	Not stated	Active ingredient, 28 ± 1C, 60±10%RH, 3 replicates of 100 sprayed, repeated 10 times, 24hr mortality	(Meled et al., 1998)
Deltamethrin 500 mg/ha	Prochloraz 25 g/ha (1:50)	Mixture potter tower	47 ±11% mortality	Winter A mellifera	Not stated	Active ingredient, 28 ± 1C, 60±10%RH, 3 replicates of 100 sprayed, repeated 10 times, 24hr mortality	(Meled et al., 1998)
Deltamethrin 62.5 mg/ha	Prochloraz 25 g/ha (1:400)	Mixture potter tower	32.5 ±3.5% mortality	Summer A mellifera	Not stated	Active ingredient, 28 ± 1C, 60±10%RH, 3 replicates of 100 sprayed, repeated 10 times, 24hr mortality	(Meled et al., 1998)
Deltamethrin 0.5, 1 ng/bee, contact	Prochloraz, difenoconazole 850 ng/bee contact (1:850-1700)	mixture	Significant increased hypothermia	A mellifera	Not stated	22°C, no other info on study design	(Vandame and Belzunces, 1998b) (Vandame and Belzunces, 1998a)
Lambda-cyhalothrin	Prochloraz ratio	mixture	Decreased	Apis	Not stated	Formulations, 25C, 75%RH mortality	(Pilling, 1992)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
dose response LD50 contact	related to application rate contact (1:50)		LD50 from 0.15 ug/bee to 0.0082 ug/bee (18.3 fold)	mellifera		24hr	
Lambda-cyhalothrin dose response LD50 contact	Prochoraz (EC) ratio related to application rate (1:50)	mixture	Dec LD50 from 0.068 ug ai/bee to 0.0075 ug ai/bee (9.1 fold)	Apis mellifera	Not stated	Formulations, 25C, 75%RH, 10 bees per replicate, 3 replicates per dose, 6 doses, mortality 24hr	(Pilling, 1992; Pilling and Jepson, 1993)
Lambda-cyhalothrin radiolabelled	Prochloraz (EC)ratio 1:50	mixture	Delayed metabolism and excretion of lambda cyhalothrin for 16hrs	Apis mellifera	Not stated	Active ingredient and formulation, 25C, 75%RH, 300 bees dosed per treatment, frass collected at 2,4,16 and 24 hrs	(Pilling et al., 1995)
Lambda-cyhalothrin	Prochloraz (ratio related to application rate) 1:132	mixture	6.7 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Lambda-cyhalothrin	Prochloraz (ratio related to application rate) 1:132	mixture	14.5 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Alphacypermethrin	Propiconazole (ratio related to application rate) 1:8	mixture	1.2 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates.	(Thompson and Folkard-Ward, 2001)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
					within 4 weeks of start of study	Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	
Alphacypermethrin	Propiconazole (ratio related to application rate) 1:9.4	mixture	2.1 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Alphacypermethrin	Propiconazole (ratio related to application rate) 1:9.4	mixture	2.5 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients,, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Lambda-cyhalothrin	Propiconazole 1:12.5	mixture	1.3 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Folkard-Ward, 2001)
Lambda-cyhalothrin	Propiconazole 1:1250	mixture	16 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Folkard-Ward, 2001)
Lambda-cyhalothrin	Propiconazole 1:50	mixture	1.6 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t	(Thompson and Folkard-Ward, 2001)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
					weeks of start of study	test vs additive toxicity	
Lambda-cyhalothrin	Propiconazole 1:6.25	mixture	1.6 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Folkard-Ward, 2001)
Lambda-cyhalothrin dose response LD50 contact	Propiconazole (EC) ratio related to application rate (1:16.6)	mixture	Dec LD50 from 0.068 ug ai/bee to 0.0042 ug ai/bee (16.2 fold)	Apis mellifera	Not stated	Formulations, 25C, 75%RH, 10 bees per replicate, 3 replicates per dose, 6 doses, mortality 24hr	(Pilling and Jepson, 1993)
Lambda-cyhalothrin	Propiconazole (ratio related to application rate) 1:33	mixture	3.0 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Lambda-cyhalothrin	Propiconazole (ratio related to application rate) 1:33	mixture	2.9 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Alphacypermethrin	Tebuconazole (ratio related to application rate) 1:19	mixture	No change in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
					start of study		
Alphacypermethrin	Tebuconazole (ratio related to application rate) 1:19	mixture	2.5 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Lambda-cyhalothrin	Tebuconazole (ratio related to application rate) 1:66	mixture	2.2 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Lambda-cyhalothrin	Tebuconazole (ratio related to application rate) 1:66	mixture	2.7 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Lambda-cyhalothrin	Thiophanate methyl (ratio related to application rate) 1:100	mixture	1.9 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Alphacypermethrin	Thiophanate methyl (ratio related to application rate) 1:67	mixture	20 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Folkard-Ward, 2001)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
Alphacypermethrin	thiophanate-methyl (ratio related to application rate) 1:34	mixture	1.2 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Alphacypermethrin	thiophanate-methyl (ratio related to application rate) 1:34	mixture	1.6 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Lambda-cyhalothrin	Thiophanate-methyl (ratio related to application rate) 1:121	mixture	3 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Lambda-cyhalothrin	thiophanate-methyl (ratio related to application rate) 1:121	mixture	1.6 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Lambda-cyhalothrin dose response LD50 contact	Triademefon (WP) ratio related to application rate (1:23.3)	mixture	Dec LD50 from 0.068 ug ai/bee to 0.0059 ug ai/bee (11.5 fold)	Apis mellifera	Not stated	Formulations, 25C, 75%RH, 10 bees per replicate, 3 replicates per dose, 6 doses, mortality 24hr	(Pilling and Jepson, 1993)
Lambda-cyhalothrin	Triadimenol (EC)	mixture	Dec LD50	Apis	Not stated	Formulations, 25C, 75%RH, 10 bees	(Pilling and Jepson, 1993)
Pesticide	**Pesticide**	**Time between treatments**	**Effect**	**Strain**	**Pre-exposure treatments**	**Design**	**Reference**
dose response LD50 contact	(ratio related to application rate (1:16.6)		from 0.068 ug ai/bee to 0.0090 ug ai/bee (7.55 fold)	mellifera		per replicate, 3 replicates per dose, 6 doses, mortality 24hr	
+ other fungicides							
alphacypermethrin	Chlorothalonil (chloronitrile) (ratio related to application rate) 1:34	mixture	2.1 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Lambda-cyhalothrin	Chlorothalonil (chloronitrile) (ratio related to application rate) 1:121	mixture	1.4 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
alphacypermethrin	Mancozeb (dithiocarbamate) (ratio related to application rate) 1:107	mixture	2 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Folkard-Ward, 2001)
Lambda-cyhalothrin	Mancozeb (dithiocarbamate) (ratio related to application rate) 1:160	mixture	4.3 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Folkard-Ward, 2001)
Other pesticides							
Pesticide	**Pesticide**	**Time between treatments**	**Effect**	**Strain**	**Pre-exposure treatments**	**Design**	**Reference**
Fluvalinate, 1 and 10 ppm oral	Carbaryl, paraquat, mancozeb 1 and 10 ppm oral	mixture	Additive toxicity	Apis mellifera (Italian)	Not stated	Formulations, 25 bees/rep, 18 and 25C, 50-70% RH, 4 repeated studies, randomised block design, 12, 24 and every 24h to 5 days after treatment, ANOVA	(Chaney, 1988)
Permethrin, 1 and 10 ppm oral	Carbaryl, paraquat, mancozeb 1 and 10 ppm oral	mixture	Additive toxicity	Apis mellifera (Italian)	Not stated	Formulations, 25 bees/rep, 18 and 25C, 50-70% RH, 4 repeated studies, randomised block design, 12, 24 and every 24h to 5 days after treatment, ANOVA	(Chaney, 1988)
Deltamethrin 12.5 ng contact	Pirimicarb 2.5 µg contact	mixture	Inc in AChE activity (soluble form in dead bees, soluble and membrane forms in live bees) resulting from deltamethrin treatment not reduced by pirimicarb	Apis mellifera	Not stated	Active ingredients, dead bees collected every 30mins for 24hr, live bees collected at 24hr, AChE activity by electrophoresis, 10 experiments in triplicate, ANOVA, paired t-test	(Badiou and Belzunces, 2008)

For only a few of the binary mixtures tested in the laboratory, also a semi-field study is available. As shown in Table 2.15, no mixture effects were detected on the endpoints assessed in these studies.

Table 2.15 gives an overview of the known semi-field studies from the scientific literature.

Table 2.15 Semi-field studies with pesticide mixtures

Pesticide	Pesticide	Acute effect	Chronic effect	Test system	Reference
Honeybee					
Thiacloprid 96, 144 g ai/ha	Tebuconazole 375 g ai/ha	No increased adult mortality	No change in hive strength	Flowering phacelia or oilseed rape	(Schmuck et al., 2003b)
Acetamiprid 168.1 g/ha	Triflumizole 280 g/ha	No effect on adult mortality		alfalfa	(Iwasa et al., 2004a)
Tau fluvalinate 48 g ai/ha	Difenoconazole 126 g ai/ha + carbendazim 250 g ai/ha	No effect on adult mortality	No effect on colony development	Flowering phacelia or oilseed rape	(Lefebvre and Bassand, 2001)
Lambda cyhalothrin	Flusilazole	Increased mortality immediately post application and days 1and 2	Decreased foraging but increased mortality compared with fungicide alone	Flowering phacelia	Defra (PN0945, 2004)
Other bee species					
Iprodione 2.24 kg Rovral/Ha),	Surfactant Dyne-Amic (0.75% v/v) + inorganic foliar feed (5% vol/vol)	No effects	No effect	Osmia lignaria, 10-14 females, 15-18 males flowering phacelia time spent inside the nest depositing pollen-nectar loads, foraging time, cell production rate, and survival, control + toxic reference (dimethoate)	(Ladurner et al., 2008)

Table 2.16 Laboratory studies with pesticide and varroacides

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
+ classical synergists							
Tau-fluvalinate	Indole-3-carbinol 1 mg/g candy oral	3 days prior	No change in LD50	Apis mellifera	Not stated	Active ingredients, 3 day old bees, 20 bees/replicate 32-34C, 24h mortality, Probit analysis	(Johnson et al., 2012)
Tau-fluvalinate	Phenobarbital 5 mg/g candy oral	3 days prior	Dec LD50 from 8050 to 190 ng/bee	Apis mellifera	Not stated	Active ingredients, 3 day old bees, 20 bees/replicate 32-34C, 24h mortality, Probit analysis	(Johnson et al., 2012)
Tau-fluvalinate	Quercetin 10 mg/g candy oral	3 days prior	Inc LD50 from 8050 to 11400 ng/bee	Apis mellifera	Not stated	Active ingredients, 3 day old bees, 20 bees/replicate 32-34C, 24h mortality, Probit analysis	(Johnson et al., 2012)
Tau-fluvalinate	Salicylic acid 2.5 mg/g candy oral	3 days prior	Dec LD50 from 8050 to 4450 ng/bee	Apis mellifera	Not stated	Active ingredients, 3 day old bees, 20 bees/replicate 32-34C, 24h mortality, Probit analysis	(Johnson et al., 2012)
Coumaphos LD50 20 ug/bee contact	DEF (carboxylesterase inhibitor) 10ug/bee contact	Synergist 1hr prior	LD50 7.3 ug/bee	Apis mellifera	Terramycin and fumadil used in colonies	Active ingredients, no temp/humidity/lighting information, mortality (incl knocked down) at 24hrs statistics probit analysis	(Johnson et al., 2009)
Coumaphos LD50 20 ug/bee contact	DEM (glutathione transferase inhibitor) 100 ug/bee contact	Synergist 1hr prior	LD50 19.9 ug/bee	Apis mellifera	Terramycin and fumadil used in colonies	Active ingredients, no temp/humidity/lighting information, mortality (incl knocked down) at 24hrs statistics probit analysis	(Johnson et al., 2009)
Coumaphos LD50 20 ug/bee contact	PBO (P450 inhibitor) 10ug/bee contact	Synergist 1hr prior	LD50 5.0 ug/bee	Apis mellifera	Terramycin and fumadil used in colonies	Active ingredients, no temp/humidity/lighting information, mortality (incl knocked down) at 24hrs statistics probit analysis	(Johnson et al., 2009)
+ EBI synergists							
Flumethrin	Carbendazim max	mixture	6.4 fold	Apis	No antibiotic	Active ingredients, 25 ±1 C,	(Thompson

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
	field application rate 4.4 mg/ml		increase in toxicity	mellifera	no varroacide within 4 weeks of start of study	65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs,	and Wilkins, 2003) (Defra PN0945)
Flumethrin	Difenconazole max field application rate 0.63 mg/ml	mixture	6.0 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Flumethrin	Flusilazole max field application rate 1 mg/ml	mixture	19.1 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Flumethrin	Prochloraz max field application rate 2.5 mg/ml	mixture	10 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Flumethrin	Propiconazole max field application rate 0.63 mg/ml	mixture	4.1 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs	(Thompson and Wilkins, 2003) (Defra PN0945)
Flumethrin	Tebuconazole max field application rate 1.25 mg/ml	mixture	3.1 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs,	Thompson & Wilkins 2003 (Defra PN0945)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
					of study		
Flumethrin	thiophanate-methyl max field application rate 2.3 mg/ml	mixture	1.9 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Flumethrin as Bayvarol strip	Carbendazim 12.5-100 mg/ml	Mixture or sequential (6hrs)	Slight increase in mortality	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 4 reps per dose, 10 bees per rep.Dose of pyrethroid causing 30% mortality Mortality at 4, 24, 48, 72 and 96hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Flumethrin as Bayvarol strip	Difenoconazole 2.6 mg/ml	Mixture or sequential (6hrs)	Slight increase in mortality	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 4 reps per dose, 10 bees per rep.Dose of pyrethroid causing 30% mortality Mortality at 4, 24, 48, 72 and 96hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Flumethrin as Bayvarol strip	Flusilazole 0.33-2.6 mg/ml	Mixture or sequential (6hrs)	Slight increase in mortality	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 4 reps per dose, 10 bees per rep.Dose of pyrethroid causing 30% mortality Mortality at 4, 24, 48, 72 and 96hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Flumethrin as Bayvarol strip	Propiconazole 2.8 mg/ml	Mixture or sequential (6hrs)	Slight increase in mortality	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 4 reps per dose, 10 bees per rep.Dose of pyrethroid causing 30% mortality Mortality at 4, 24, 48, 72 and 96hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Flumethrin as Bayvarol strip	Tebuconazole 4.9 mg/ml	Mixture or sequential	Slight increase in mortality	Apis mellifera	No antibiotic no varroacide	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 4 reps per	(Thompson and Wilkins,

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
		(6hrs)			within 4 weeks of start of study	dose, 10 bees per rep.Dose of pyrethroid causing 30% mortality Mortality at 4, 24, 48, 72 and 96hrs,	2003) (Defra PN0945)
Flumethrin as Bayvarol strip	thiophanate-methyl 50-100 mg/ml	Mixture or sequential (6hrs)	Slight increase in mortality	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 4 reps per dose, 10 bees per rep.Dose of pyrethroid causing 30% mortality Mortality at 4, 24, 48, 72 and 96hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Fluvalinate	Carbendazim max field application rate 4.4 mg/ml	mixture	3 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients,, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Fluvalinate	Difenconazole max field application rate 0.63 mg/ml	mixture	2.5 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients,, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Fluvalinate	Flusilazole max field application rate 1 mg/ml	mixture	19 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Fluvalinate	Prochloraz max field application rate 2.5 mg/ml	mixture	53 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
Fluvalinate	Propiconazole max field application rate 0.63 mg/ml	mixture	2.5 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients,, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs, Students t test vs additive toxicity	(Thompson and Wilkins, 2003) (Defra PN0945)
Fluvalinate	Tebuconazole max field application rate 1.25 mg/ml	mixture	6.1 fold increase in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Fluvalinate	thiophanate-methyl max field application rate 16.2 mg/ml	mixture	1.9 fold decrease in toxicity	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Active ingredients, 25 ±1 C, 65±5%RH, OECD contact method, 3 reps per dose, 10 bees per rep 5 dose rates. Mortality at 4, 24, 48hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Fluvalinate as Apistan strip	Carbendazim 100 mg/ml	Mixture or sequential (6hrs)	No change in mortality	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 4 reps per dose, 10 bees per rep.Dose of pyrethroid causing 30% mortality Mortality at 4, 24, 48, 72 and 96hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Fluvalinate as Apistan strip	Difenoconazole 2.6 mg/ml	Mixture or sequential (6hrs)	No change in mortality	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 4 reps per dose, 10 bees per rep.Dose of pyrethroid causing 30% mortality Mortality at 4, 24, 48, 72 and 96hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Fluvalinate as Apistan strip	Flusilazole 0.03-2.6 mg/ml	Mixture or sequential (6hrs)	increased mortality	Apis mellifera	No antibiotic no varroacide within 4	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 4 reps per dose, 10 bees per rep.Dose of	(Thompson and Wilkins, 2003) (Defra

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
					weeks of start of study	pyrethroid causing 30% mortality Mortality at 4, 24, 48, 72 and 96hrs,	PN0945)
Fluvalinate as Apistan strip	Propiconazole 2.8 mg/ml	Mixture or sequential (6hrs)	No change in mortality	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 4 reps per dose, 10 bees per rep.Dose of pyrethroid causing 30% mortality Mortality at 4, 24, 48, 72 and 96hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Fluvalinate as Apistan strip	Tebuconazole 4.9 mg/ml	Mixture or sequential (6hrs)	No change in mortality	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 4 reps per dose, 10 bees per rep.Dose of pyrethroid causing 30% mortality Mortality at 4, 24, 48, 72 and 96hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
Fluvalinate as Apistan strip	thiophanate-methyl 12.5-100 mg/ml	Mixture or sequential (6hrs)	Inc mortality	Apis mellifera	No antibiotic no varroacide within 4 weeks of start of study	Formulations, 25 ±1 C, 65±5%RH, OECD contact method, 4 reps per dose, 10 bees per rep.Dose of pyrethroid causing 30% mortality Mortality at 4, 24, 48, 72 and 96hrs,	(Thompson and Wilkins, 2003) (Defra PN0945)
+ other pesticides/varroacides							
Fluvalinate LD50 6.75 ug/bee contact	Coumaphos 0.1, 0.3, 1, 3, 10 ug/bee contact	Synergist 1hr prior	LD50 6.14, 3.29, 2.68, 1.53, 0.21	Apis mellifera	Terramycin and fumadil used in colonies	Active ingredients, no temp/humidity/lighting information, mortality (incl knocked down) at 24hrs statistics probit analysis	(Johnson et al., 2009)
Coumaphos LD50 20 ug/bee contact	Fluvalinate 0.1, 0.3, 1, 3 ug/bee contact	Synergist 1hr prior	LD50 20.9, 12.8, 6.1, 6.1 ug/bee	Apis mellifera	Terramycin , Apilife Var (Thymol plus Eucalyptus Oil, Menthol and	Active ingredients, no temp/humidity/lighting information, mortality (incl knocked down) at 24hrs statistics probit analysis	(Johnson et al., 2009)

Pesticide	Pesticide	Time between treatments	Effect	Strain	Pre-exposure treatments	Design	Reference
					Camphor) and fumadil used in colonies		
Fluvalinate (queen tabs)	Bifenthrin contact	48h preexposure	1.9 fold inc in toxicity	Apis mellifera	Not stated	20 C in dark, Six replicates of 10 bees, six dose levels 24hr mortality, Probit analysis	(Ellis et al., 1997)
Fluvalinate (queen tabs)	Carbaryl contact	48h preexposure	No change in toxicity	Apis mellifera	Not stated	20 C in dark, Six replicates of 10 bees, six dose levels 24hr mortality, Probit analysis	(Ellis et al., 1997)
Fluvalinate (queen tabs)	Methyl parathion contact	48h preexposure	No change in toxicity	Apis mellifera	Not stated	20 C in dark, Six replicates of 10 bees, six dose levels 24hr mortality, Probit analysis	(Ellis et al., 1997)

Table 1. List of synergisms of xenobiotics in honeybee Apis mellifera and the proposed mechanisms (taken from Glavan et al. 2013).

Xenobiotic	Xenobiotic (P450 inhibitor)	Reference
Mechanism of synergy: inhibition of P450 detoxifying enzymes		
pyrethroid insecticides	*classical P450-inhibitor*	
cyfluthrin	piperonyl butoxide	(Johnson et al. 2006)
permethrin	piperonyl butoxide	(Hagler et al. 1989)
lambda-cyhalothrin	piperonyl butoxide	(Johnson et al. 2006)
tau-fluvalinate	piperonyl butoxide	(Johnson et al. 2006; Johnson et al. 2013)
neonicotinoid insecticides	*classical P450-inhibitor*	
imidacloprid	piperonyl butoxide	(Iwasa et al., 2004, Johnson et al. 2012)
acetadimiprid	piperonyl butoxide	(Iwasa et al. 2004)
thiacloprid	piperonyl butoxide	(Iwasa et al. 2004)
carbamate insecticide	*classical P450-inhibitor*	
carbaryl	piperonyl butoxide	(Georghiou and Atkins Jr. 1964)
hive varroacides	*classical P450-inhibitor*	
tau-fluvalinate	piperonyl butoxide	(Johnson et al. 2009, Johnson et al. 2013)
coumaphos	piperonyl butoxide	(Johnson et al. 2009, Johnson et al. 2013)
fenpyroximate	piperonyl butoxide	(Johnson et al., 2013)
neonicotinoid insecticides	*EBI (ergosterol biosynthesis inhibitor) fungicides*	
acetamiprid	epoxiconazole, propiconazole, triadimefon, triflumizole, uniconazole-P	(Iwasa et al. 2004)
thiacloprid	prochloraz, propiconazole, tebuconazole, triflumizole	(Schmuck et al. 2003, Iwasa et al. 2004)
imidacloprid	propiconazole, triflumizole	(Iwasa et al. 2004)
pyrethroid insecticides	*EBI (ergosterol biosynthesis inhibitor) fungicides*	
deltamethrin	difenoconazole+carbendazim, prochloraz, prochloraz+ difenoconazole 850	(Belzunces and Colin 1993, Colin and Belzunces 1992, Papaefthimiou and Theophilidis 2001, Vandame and Belzunces1998b, Vandame and Belzunces 1998a)
lambda-cyhalothrin	difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole, thiophanate-methyl	(Thompson and Wilkins 2003)
alphacypermethrin	difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole	(Thompson and Wilkins 2003)
hive varroacides	*EBI (ergosterol biosynthesis inhibitor) fungicides*	
coumaphos	prochloraz	(Johnson et al. 2013)
flumethrin	carbendazim, difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole, thiophanate-methyl	(Thompson and Wilkins 2003)

Xenobiotic	Xenobiotic (P450 inhibitor)	Reference
tau-fluvalinate	carbendazim, difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole, thiophanate-methyl, myclobutanil, metconazole, fenbuconazole,	(Thompson and Wilkins 2003, Johnson et al. 2013)
fenpyroximate	prochloraz	(Johnson et al. 2013)
hive varroacides	*hive varroacides*	
coumaphos	tau-fluvalinate	(Johnson et al. 2009, 2013)
thymol	tau-fluvalinate, coumaphos	(Johnson et al. 2013)
amitraz	tau-fluvalinate, coumaphos, fenpyroximate	(Johnson et al. 2013)
fenpyroximate	tau-fluvalinate, coumaphos	(Johnson et al. 2013)
Mechanism of synergy: increased oxidative stress		
hive varroacides	*Fungicides (mitochondrial inhibitors)*	
tau-fluvalinate	pyraclostrobin, boscalid	(Johnson et al. 2013)
fenpyroximate	pyraclostrobin	(Johnson et al. 2013)
Unknown mechanism of synergy		
oxalic acid	tau-fluvalinate, fenpyroximate, amitraz, thymol	(Johnson et al. 2013)
herbicide atrazine	carbamate insecticides (carbaryl, carbofuran)	(Sonnet et al. 1978)
thio and dithiophosphoric ester pesticides – ethyl parathion, dimethoate, dialifos	coumaphos varroacide	(Lienau 1990)
thiacloprid (neonicotionoid)	fungicides cyprodinil, tolyfluanid	(Schmuck et al. 2003)
alphacypermethrin, lambda-cyhalothrin	fungicide chlorothalonil fungicide chlorothalonil	(Thompson and Wilkins 2003)

For other organisms, mixture interactions have been described as well. Examples of synergistic effects between pesticides in aquatic organisms are given in Table 6.4, taken from Verbruggen and van den Brink, 2010).
(EFSA 2012;10(5):2668) Both the WiGRAMP report (COT, 2002) and Verbruggen and van den Brink (2010) suggest that synergistic and antagonistic interactions are rarely observed but there are some exceptions.

Table 6.4: Examples of combinations resulting in synergism (from Verbruggen and van den Brink, 2010)

Compounds in mixture	Mode/Site of action	Species tested	Deviation from Concentration addition
Pirimicarb/monocrotophos	Similar	*Tilapia nilotica*	2.7-fold
Quinalphos/phenthoate	Similar	*Oreochromis*	10-fold

		mossambicus	
Malathion dioxathion	Similar	*Salmo gairdneri*	8.2-fold
Carbaryl/phenthoate	Similar	*Channa punctatus*	2.2-fold
Atrazine/trichlorfon	Dissimilar	*Chironomus tentans*	3.8-fold
Atrazine/malathion	Dissimilar	*Chironomus tentans*	2.8-fold
Deltamethrin/carbaryl	Dissimilar	*Lymnaea acuminata*	20-fold
Anilazine/tri-allate	Dissimilar	*Chlorella fusca*	3.5-fold

EFSA 2012;10(5):2668):

"Organophosphorus esters and carbamates

Laetz et al. (2009) assessed the combined effects of organophosphorus esters (diazinon, malathion, chlorpyrifos) and carbamates (carbaryl, carbofuran) on the Coho salmon and showed significant synergism through increased brain AChE inhibition following exposure to mixtures of organophosphates or organophosphates and carbamates resulting in some cases in death of the fish (chlorpyrifos + malathion and diazinion + malathion). The basis for synergism between these pesticides would be expected to be a toxicodynamic interaction at the target receptor (cholinesterase).

In addition, organophosphates have been used in a targeted approach to break resistance associated with pyrethroids; in combination the organophosphates appear to inhibit P 450 metabolism of certain pyrethroids resulting in an increased toxicity through a toxicokinetic interaction (Ahmed, 2009).

Herbicides and insecticides

Mixtures of herbicides and insecticides can also act synergistically. The herbicide atrazine in binary mixtures with the insecticides chlorpyrifos, diazinon and methyl parathion showed synergism in the amphipod *Hyalella azteca* (Anderson and Lydy, 2002). The mechanism of action was proposed to be induction of the P450 by atrazine increasing the rate of conversion of the parent thion to their active oxon forms resulting in increased AChE inhibition.

The interaction of herbicides with insecticides has also been reported in gibberellin inhibitor plant growth regulators (e.g. flurprimidol, paclobutrazol and triexapac-ethyl) which have also been identified as P450 inhibitors (Ramoutar et al., 2010) and synergize the activity of pyrethroids in coleoptera. Therefore mixtures of herbicides which interact by inducing or inhibiting P450s with pesticides which are also activated or metabolized by these enzymes may result in synergism of toxicity.

EBI fungicides and insecticides

The EBI (ergosterol biosynthesis inhibiting) fungicides are widely reported to inhibit vertebrate and invertebrate P450s and include major classes such as the Demethylation Inhibitors (DMI)-fungicides (imidazoles, triazoles, piperazines, pyrimidines, pyridines) and the amines (piperidines, morpholines, spiroketalamines). The toxicity of these compounds has been extensively reviewed in Thompson (1996). More recently synergism between EBI fungicides and neonicotinoid insecticides (thiacloprid + propiconazole) has been reported (Iwasa et al., 2004) but this has not been demonstrated at field realistic rates following sequential applications (Schmuck et al., 2003).

Recently, the joint effects of chemical mixtures on the life-history traits of *Daphnia magna* Straus were investigated. For instance imidacloprid was tested together with thiacloprid and imidacloprid with nickel chloride. For the mixture exposure of imidacloprid and thiacloprid, a synergistic pattern was observed in sub-lethal doses (number of neonates produced), while for the body length the best fit was shown with the CA model. In the mixture exposure of imidacloprid and nickel, no deviation from the IA was observed for the neonate production data; for the body length parameter, a synergistic pattern was observed in low doses of the chemicals (Pavlaki et al., 2011).

Recently, Bjergager et al. (2011) investigated the magnitude of the synergism between the conazole fungicide prochloraz and the pyrethroid (esfenvalerate) at environmentally realistic concentrations onzooplankton and phytoplankton at days 0, 1, 2, 4, 7, 14, 21, and 28 after pesticide application by comparing EC20-values estimated on the basis of concentration–response curves for days 2, 4, and 7.
Hence, prochloraz was shown to enhance the toxicity of esfenvalerate four to six fold for copepods and three to sevenfold for cladocerans with an indication of stabilisation or the beginning of recovery between day 7 and day 14 and full recovery in some of the less affected populations of cladocerans, copepods, and chironomids after 28 days. Authors concluded that the occurrence of the synergistic interactions between prochloraz and esfenvalerate in the microcosms and at environmentally realistic
concentrations implies that the synergistic interactions may also take place in invertebrate communities in natural ponds and ditches being exposed to azoles and pyrethroids via for example runoff or drift.

Synergistic toxicity of pesticides has been measured in a number of terrestrial athropods and annelids and nematods. For example, the synergy of atrazine and organophosphate insecticides has been demonstrated in midges (*Chironomus tentans*) (Pape-Lindstrom and Lydy 1997). In the earthworm, *Eisenia fetida,* atrazine and cyanazine increased the toxicity of chlorpyrifos 7.9- and 2.2-fold and body residue analysis suggesting that the greater-than-additive response may be due to increased biotransformation to more toxic oxon metabolites (Lydy and Linck, 2003). Recently, statistically significant dose-dependent synergism was also shown in the nematode *C. elegans* ($P<0.01$) whereas concentration addition was measured on *E. fetida* after exposure to similarly acting neonicotinoid pesticides imidacloprid and thiacloprid.

Reproductive toxicity of 10 binary mixtures of five different pesticides from three classes of neurotoxic pesticides with the same MOA (neuroexcitation) but different molecular mechanisms were tested in binary mixtures with the nematode *Caenorhabditis elegans*. Both CA and IA were found to be valid models for prediction of the toxicity of 4 of the mixtures, however, evidence for interaction was found in the remaining six cases and could be explained by toxicokinetics-interaction i.e. production of a metabolically activated or a metabolically deactivated chemical and/or cases where the relative potencies of the two tested chemicals differed greatly.

Finally, synergistic effects of insecticides (bifenthrin, imidacloprid) on tawny mole cricket (*Scapteriscus vicinus Scudder*). LD_{50} values for bifentrin and imidacloprid increased by 3.8- and 8.8-fold respectively in adults and 1.5 and 19-fold in nymphs.

Synergistic interactions between natural stressors and chemicals were reported in more than 50% of the available studies on these interactions. Antagonistic interactions were also detected, but in fewer cases (Holmstrup et al., 2010)."

Bees can be exposed to pesticides in many different ways. For now, we will only focus on residues found in pollen, bee bread, honey, comb wax and honey bees themselves. Table G1 gives an overview of the available data from the scientific literature concerning the amount of residues found in pollen, honey, comb wax and honey bees. Table G6 gives an overview of the pesticides found in bee bread, found during a monitoring survey. Table 1.10 presents an overview of pesticide residues found in bee bread taken from the scientific literature.

Table G1: Residues in pollen, honey, comb wax and honey bees from the scientific literature

Pesticide	Pollen loads			Honey			Comb wax			Honey bees			
	Max (µg/kg)	LOD/LOQ (µg/kg)	Ref	Max (µg/kg)	LOD/LOQ (µg/kg)	Ref	Max (µg/kg)	LOD/LOQ (µg/kg)	Ref	Max (µg/kg)	LOD/LOQ (µg/kg)	Ref	
6-Chloronicotinic acid	9.3	0.2/0.6	A	10.2	0.3/0.6	A				1.7	0.3/0.6	A	Neo
Acrinathrin				2400		2	590		1				
Aldicarb	ND	5.0/10.0	A	ND	3.5/10.0	A				15.3	5.0/10.0	A	Carb
Aldicarb sulfon	ND	5.0/10.0	A	ND	3.5/10.0	A				21	5.0/10.0	A	Carb
Aldicarb sulfoxide	ND	5.0/10.0	A	ND	3.5/10.0	A				19.2	5.0/10.0	A	Carb
Aldrin				150		4							Cyc
Amitraze I	115	46.3/69.4	B	26	10.0/37.0	B				30	18.5/27.8	B	
Amitraze II	129	8.1/17.3	B	116	0.3/4.3	B				40	4.3/10.8	B	
Atrazine				81		5							
Azinphos ethyl										94		6	OP
Azinphos-methyl	ND	57.0/196.7	A	55.3	5.5/20.0	A	817	5.0/10.0	A	91		6	OP
Benalaxyl	ND	21.3/42.7	B	ND	5.7/14.2	B				5.7<R<28.4	5.7/28.4	B	
Bitertanol				0.1		8							
Bromophos ethyl				12		10							OP
Bromophos methyl										1733		6	OP
Bromopropylate				245		12	135,000		11	2245		12	
Bupirimate	2.8<R<21.4	2.8/21.4	B	5.7<R<14.2	5.7/14.2	B				ND	5.7/14.2	B	
Buprofezine	ND	29.9/59.9	B	43	23.9/35.9	B				ND	23.9/71.8	B	
Captan				19		14							
Carbaryl	94,000		15	31.3	3.5/10.0	A				214.3	5.7/10.0	A	Carb
Carbaryl	276.9	5.0/10.0	A	0.1<R<3.8	0.1/3.8	B				0.4<R<3.8	0.4/3.8	B	Carb
Carbaryl	15	0.7/1.2	B										Carb
Carbendazim	2595	0.1/1.0	B	88	0.5/4.0	B				66	0.6/4.0	B	
Carbofuran	2	0.4/1.0	B	645		17				669		6	Carb
Carbofuran	137.5	5.0/10.0	A	0.03<R<3.8	0.03/3.8	B				ND	0.1/3.8	B	Carb
Carbofuran				35.5	3.5/10.0	A				14.9	5.7/10.0	A	Carb
Chlorfenvinphos				0.2		18	7620		1				
Chlorpyrifos	140	8.0/20.0	B	15		5				57		6	OP
Chlorpyrifos				ND	3.2/8.0	B				180	0.8/3.2	B	OP
Chlorpyrifos ethyl	35	10.0/34.5	A	ND	3.5/10.0	A	19		A	ND	10.0/34.5	A	OP
Chlorpyriphos-methyl	ND	1.3/19.5	B	0.2		18				36		6	OP
Chlorpyriphos-methyl				0.1<R<5.2	0.1/5.2	B				ND	0.3/5.2	B	OP
Coumaphos	40	4.6/18.4	B	2020		21	4112	5.0/10.0	A	2777		6	
Coumaphos	1700	37.0/142.6	A	29	3.7/9.2	B				47	0.4/3.7	B	
Coumaphos				934	3.5/10.0	A				24 840	37.0/142.8	A	
Cyfluthrin	ND	7.0/98.7	A				158	5.0/10.0	A	ND	7.0/39.5	A	Pyr
Cymiazole				17		24							
Cypermethrin	1900		15	92		5	76.3	5.0/10.0	A	ND	3.8/32.7	A	Pyr
Cypermethrin	ND	3.8/93.3	A	4.5<R<37.6	4.5/37.6	B				49	4.5/27.1	B	Pyr
Cypermethrin	ND	56.4/169.1	B										Pyr
Cyproconazole	8		15	ND	3.5/10.0	A				ND	5.0/10.0	A	
Cyproconazole	5<R<10	5.0/10.0	A	4	4.0/10.1	B				ND	2.0/10.1	B	
Cyproconazole	22	10.1/50.4	B										
DDT-p,p''				658		17							OC
Deltamethrin	39	0.1/29.9	A	2.7	5.0/20.0	A	14.7	5.0/10.0	A	43	0.1/24.9	A	Pyr
Dialifos				92		4							OP
Diazinon	ND	10.5/26.3	B	35		24				6.3<R<14.7	6.3/14.7	B	OP
Diazinon				14	7.4/10.5	B							OP
Dichlofluanid				11		26							
Dieldrin	9.8<R<24.6	9.8/24.6	B	13		4				ND	3.9/9.8	B	Cyc
Dieldrin				ND	3.9/29.5	B							Cyc
Diethofencarb	3	0.6/1.9	B	0.04<R<3.8	0.04/3.8	B				ND	0.2/3.8	B	
Difenoconazole	411		14	0.9		14							
Dimethoate	ND	18.0/59.6	A	ND	13.6/18.2	B				ND	18.0/59.6	A	OP
Dimethoate	9.1<R<45.4	9.1/45.4	B							ND	3.6/27.3	B	OP

Endosulfan	340	0.1/8.0	A	24		5	243.1	5.0/10.0	A	17	0.1/8.0	A	OC
Endosulfan				ND	3.5/10.0	A							OC
Endosulfan I													OC
Endosulfan II	ND	15.5/51.5	B	10.3<R<30.9	10.3/30.9	B				ND	10.3/30.9	B	OC
Endrin				7		4							Cyc
Epoxyconazole	ND	5.0/10.0	A	ND	3.5/10.0	A				13.7	5.0/10.0	A	
Fenitrothion	ND	19.0/66.9	A	ND	3.5/10.0	A	511	5.0/10.0	A	10,330		6	OP
Fenoxycarb	ND	1.0/3.3	B	0.1<R<4.1	0.1/4.1	B				157		6	IGR
Fenoxycarb										20	0.6/4.1	B	IGR
Fenpropathrin													Pyr
Fenthion	ND	8.0/30.6	A	ND	3.5/10.0	A	ND	5.0/10.0	A	38		6	OP
Fipronil	0.3<R<0.5	0.3/0.5	A	ND	0.3/1.3	A				0.7	0.3/0.5	A	
Fipronil desulfinyl	1.5	0.3/0.5	A	ND	0.3/1.3	A				2.5	0.3/0.5	A	
Fipronil sulfon	3.7	0.3/0.5	A	ND	0.3/1.3	A				0.6	0.3/0.5	A	
Flumethrin	50		28	1		28	50		28				
Flusilazole	71		15	0.03		8				18	5.0/10.0	A	
Flusilazole	71.0	5.0/10.0	A	ND	3.5/10.0	A				2.1<R<10.3	2.1/10.3	B	
Flusilazole	52	3.6/15.5	B	4.1<R<10.3	4.1/10.3	B							
Fluvalinate				750		24							
Fluvalinate (tau-Fluvalinate)	2,020	1.1/76.0	A	44.7	3.5/10.0	A	446	5.0/10.0	A	326	1.1/11.4	A	
Fluvalinate (tau-Fluvalinate)	85	4.6/22.8	B	30	3.7/9.1	B				53	3.7/9.1	B	
Heptachlor				57		4							Cyc
Heptenophos				230		17				162		6	OP
Hexachlorobenzene				270		17							
Hexaconazole	12		15	ND	3.5/20.0	A				22.7	7.5/10.0	A	
Hexaconazole	106	7.5/10.0	A										
Hexythiazox	ND	4.8/10.2	B	0.1<R<4	0.1/4.0	B				0.8<R<3.9	0.8/3.9	B	Acar
Imazalil	ND	6.9/25.5	B	0.7<R<4.1	0.7/4.1	B				ND	1.4/10.2	B	
Imidacloprid	5.7	0.2/1.0	A	2		29				11.1	0.3/1.0	A	Neo
Imidacloprid	2.6<R<12	2.6/12.0	B	1.8	0.3/1.0	A				ND	0.4/9.6	B	Neo
Imidacloprid	18*	0.1/1.0	D	0.2<R<3.9	0.2/3.9	B							Neo
Iprodione	5511		30	266		30				ND	9.7/19.5	B	
Iprodione	15.6<R<48.7	15.6/48.7	B	ND	9.7/19.5	B							
Lambda-cyhalothrin				10.3		A				47	0.4/12.9	A	Pyr
Lindane	7		29	4310		17	290		1	11		29	OC
Lindane	9.0	0.1/4.0	A	10.3		A	32.2	5.0/10.0	A	17.4	0.1/1.5	A	OC
Malathion	ND	9.0/31.5	A	243		5	6000		31	ND	9.0/31.5	A	OP
Malathion				ND	3.5/10.0	A	18.1	5.0/10.0	A				OP
Mercaptodimethur	ND	5.0/10.0	A	ND	3.5/10.0	A				27	5.6/10	A	Anti-ulog
Mercaptodimethur sulfon	ND	5.0/10.0	A	ND	3.5/10.0	A				11.5	5.0/10.0	A	Anti-ulog
Mercaptodimethur sulfoxide	ND	5.0/10.0	A	ND	3.5/10.0	A				ND	5.6/10.0	A	Anti-ulog
Metamidophos										38		6	OP
Methidathion	ND	13.0/49.6	A	68		17	ND	5.0/10.0	A	ND	13.0/49.6	A	OP
Methiocarb				27		17				346		6	Carb
Methoxychlor				593		4							OC
Mevinphos	ND	3.8/27.7	A	ND	3.5/10.0	A	204	5.0/10.0	A	ND	3.8/18.5	A	OP
Myclobutanil	20.3	5.0/10.0	A	ND	3.5/10.0	A				29.2	5.0/10.0	A	
Oxamyl	38.4	5.0/10.0	A	ND	3.5/10.0	A				ND	5.0/10.0	A	Carb
Parathion ethyl	19		15				99		7	ND	8.0/30.4	A	OP
Parathion ethyl	8<R<30.4	8.0/30.4	A	3.5<R<10	3.5/10.0	A	5		6				OP
Parathion ethyl							99	5.0/10.0	A				OP
Parathion methyl	10<R<39.5	10.0/39.5	A	50		33							OP
p-Dichlorobenzene				112		25	60 000		25				OC
Penconazole	126		15							5<R<10	5.0/10.0	A	
Penconazole	126.0	5.0/10.0	A	ND	3.5/10.0	A				8		29	

Phenthoate										1		6	OP
Phorate				0.9		18							OP
Phosalone	ND	10.2/15.4	B	ND	4.1/10.2	B				4.1<R<10.2	4.1/10.2	B	OP
Phosmet	78	14.8/24.6	B	42	3.9/9.8	B				96		6	OP
Phosmet										62	9.8/19.7	B	OP
Phosphamidon										50		6	OP
Phoxim	ND	2.7/15.5	B	0.1<R<7.3	0.1/7.3	B				355		6	OP
Piperonyl butoxide	ND	9.0/45.2	B	3.6<R<9	3.6/9.0	B				1.1<R<3.6	1.1/3.6	B	Syn
Pirimiphos ethyl										30		6	OP
Pirimiphos methyl				19		10							OP
Prochloraz	ND	4.9/14.8	B	0.2<R<11.4	0.2/11.4	B				ND	0.7/4.6	B	
Procymidone							27.7	5.0/10.0	A				
Profenofos										17		6	OP
Propiconazole	ND	4.3/85.1	B	ND	11.1/42.5	B				2.6<R<17.0	2.6/17.0	B	
Pyrazophos				6		10				53		6	OP
Pyriproxyfen	10.7<R<21.5	10.7/21.5	B	7.5<R<10.7	7.5/10.7	B				2.1<R<4.3	2.1/4.3	B	IGR
Quinalphos										70		6	OP
Simazine				17		5							
Tebuconazole	5		5	ND	3.5/20.0	A				31.1	10.0/20.0	A	
Tebuconazole	33.2	10.0/20.0	A	12.8<R<25.8	12.8/25.8	B				ND	5.1/17.9	B	
Temephos				7		10				689		6	OP
Tetraconazole	ND	5.0/10.0	A	ND	3.5/10.0	A				17		29	
Tetraconazole										31.3	5.0/10.0	A	
Thiophanate-methyl	3 674	16.5/51.5	B	5	0.3/10.3	B				2 419	4.1/10.3	B	
Triallate				4		26							
Triazophos										9		6	OP
Trifloxystrobin				0.3		8							
Triphenylphosphate	0.5<R<9.3	0.5/9.3	B	0.7<R<9.3	0.7/9.3	B				62	0.4/9.3	B	
Vamidothion										24		6	OP
Vinclozolin	31,909		30	173		30	21.5	5.0 10.0	A	ND	4.0/10.1	B	
Vinclozolin	70	1.5/12.6	B	109.4	3.5/10.0	A							

A: Chauzat et al., 2011
B: Wiest et al., 2011
C: Other data from European countries cited in the review article of Johnson et al., 2010:
1, Jimenez et al. (2005); 2, Bernal et al. (2000); 4, Fernandez-Muino et al. (1995);
6, Ghini et al. (2004); 7, Chauzat and Faucon (2007); 8, Nguyen et al. (2009);
10, Blasco et al. (2008); 11, Bogdanov et al. (1998); 12, Lodesani et al. (1992);
14, Kubik et al. (2000); 15, Chauzat et al. (2006); 17, Blasco et al. (2003);
18, Balayiannis and Balayiannis (2008); 21, Martel et al. (2007);
24, Fernandez et al. (2002); 25, Bogdanov et al. (2004);
26 Albero et al. (2004); 28, Bogdanov (2006); 29, Chauzat et al. (2009);
30, Kubik et al. (1999); 31, Thrasyvoulou and Pappas (1988).
D: Bonmatin et al., 2005. *These data come from pollen taken directly on the flower (maize)

ND = not detected

Varroacide
Insecticide
Fungicide
Herbicide

Systemic

Acar.: acaricide
Car.: carbamate
Cyc.: cyclodiene
IGR: insect growth regulator
Miti.: miticide
Neo.: neonicotinoid
OC: organochlorine
OP: organophosphate
PGR: plant growth regulator
Pyr.: pyrethroid
Syn.: synergist

Thompson 2012:

There are two main sources of information on the levels of pesticides being returned to the hive, pollen and nectar returned to and stored within the hive and honeybees collected from the hive. Although many reports of residues in pollen being returned to the hive by foragers are published the majority of these are based on individual pesticide residues rather than assessments of the total pesticide residue levels (see Chapter 1). The primary source of information identified was the data reported by (Chauzat et al., 2011) which showed 37.8% of all pollen samples collected from 120 hives from 24 apiaries at 5 sites across France (main types of honey were chestnut, oilseed rape, sunflower, and local mixed flower honey) contained at least two different pesticide residues with 22.2, 12.7, 2.4, and 0.5% containing two, three, four, or five different residues, respectively. 14.7% of all honeybee samples contained at least two pesticides with two (11.2%), three (2.3%), four (1.0%), or five (0.2%) active ingredients.

In addition the following was delivered to the CTGB for the re-assessment of thiacloprid and acetamiprid (project B11):

Table 7. Taken from (Pohorecka, Skubida et al. 2012)

Neonicotinoids incidence and level of their concentration (ng/g) in analyzed samples collected from winter and spring oilseed rape (2010, 2012)

active substance	Samples of nectar (∑ samples of nectar from flowers and combs, honey) n=212				Samples of pollen (∑ samples of pollen loads and bee bread) n=205			
	% positive	mean	max.	median	% positive	mean	max.	median
imidacloprid	21	0.6	2.0	0.6	0	0	0	0
clothianidin	17	2.3	10.1	1.6	11	1.8	3.7	1.2
thiamethoxam	65	4.2	12.9	3.1	37	3.8	9.9	2.9
acetamiprid	51	2.4	13.3	1.1	45	4.1	26.1	2.5
thiacloprid	64	6.5	208.8	2.5	62	89.1	1002.2	4.1

Since controls were contaminated, the study is not reliable. However, it can be noted that regardless of the residues measured, the 2010 hives overwintered without problems (overwintering was not studied in 2012).

The following table is taken from the EFSA scientific opinion (2012) 10(5):2668 :

Table G6: Residue table: data from Germany (Deutsches Bienenmonitoring; 5.1.2.e personal communication)

	Bee bread (stored pollen)			
Pesticide	Max (µg/kg)	LOD*	LOQ*	Year
Acetamiprid	2<R<5	2	5	2005/2006
	0	2	5	2007
	0	1	3	2009
Azoxystrobin	1776	2	5	2005/2006
	223	2	5	2007
	52	1	3	2009
Bitertanol	90	5	15	2005/2006
	0	5	15	2007
	0	5	15	2009
Boscalid	140	2	5	2005/2006
	928	2	5	2007
	143	1	3	2009
Bromopropylate	24	5	15	2005/2006
	18	5	15	2007
	37	5	15	2009
Chloridazon	5	2	5	2005/2006
	3<R<10	3	10	2007
	0	5	10	2009
Clofentezin	5<R<15	5	15	2005/2006
	0	10	30	2007
	no data	–	–	2009
Clothianidin	0	1	3	2005/2006
	0	1	3	2007
	1	1	3	2009
Coumaphos	135	1	3	2005/2006
	140	1	3	2007
	54	5	15	2009
Cymoxanil	3<R<10	3	10	2005/2006
	0	3	10	2007
	0	3	10	2009
Cyproconazole	25	5	15	2005/2006
	0	5	15	2007
	0	5	15	2009
Cyprodinil	132	2	5	2005/2006
	no data	2	5	2007
	1092	1	3	2009
Difenconazole	5<R<15	5	15	2005/2006
	49	5	15	2007
	410	5	15	2009
Dimethoat	20	2	5	2005/2006
	32	2	5	2007
	2	1	3	2009
Dimethomorph	12	3	10	2005/2006

	0	3	10	2007
	47	1	3	2009
Dimoxystrobin	no data	–	–	2005/2006
	no data	–	–	2007
	129	1	3	2009
Diphenylamine	0	5	15	2005/2006
	39	5	15	2007
	139	5	15	2009
Epoxiconazole	0	5	15	2005/2006
	240	5	15	2007
	0	0	15	2009
Ethofumesat	26	2	5	2005/2006
	9	2	5	2007
	6	2	5	2009
Fenpropimorph	5<R<15	5	15	2005/2006
	517	2	5	2007
	10	5	15	2009
Fenpyroximat	0	2	5	2005/2006
	97	2	5	2007
	0	2	5	2009
Fludioxonil	395	5	15	2005/2006
	561	5	15	2007
	2800	5	15	2009
Flusilazol	93	5	15	2005/2006
	69	5	15	2007
	93	5	15	2009
Imidacloprid	0	1	3	2005/2006
	3	1	3	2007
	0	1	3	2009
Indoxacarb	0	5	15	2005/2006
	51	5	15	2007
	0	2	5	2009
Iprodion	36	5	15	2005/2006
	160	5	15	2007
	23	2	5	2009
Iprovalicarb	1<R<3	1	3	2005/2006
	9	1	3	2007
	21	1	3	2009
Isoproturon	6	2	5	2005/2006
	25	2	5	2007
	11	2	5	2009
Lambda-cyhalothrin	5<R<15	5	15	2005/2006
	17	5	15	2007
	0	5	15	2009
Metalaxyl	2<R<5	2	5	2005/2006
	0	2	5	2007
	10	2	5	2009
Metamitron	9	3	10	2005/2006
	13	3	10	2007
	no data	–	–	2009

Methiocarb	0	5	15	2005/2006
	14	2	5	2007
	11	1	3	2009
Methoxyfenozid	0	1	3	2005/2006
	4	1	3	2007
	0	1	3	2009
Metobromuro	2<R<5	2	5	2005/2006
	0	2	5	2007
	0	2	5	2009
Metolachlor	29	2	5	2005/2006
	18	2	5	2007
	7	2	5	2009
Metoxuron	2<R<5	2	5	2005/2006
	0	2	5	2007
	0	2	5	2009
Metribuzin	141	5	15	2005/2006
	0	3	10	2007
	91	3	10	2009
Myclobutanil	17	2	5	2005/2006
	120	2	5	2007
	28	1	3	2009
Penconazole	0	5	15	2005/2006
	5<R<15	5	15	2007
	0	2	5	2009
Pendimethalin	5<R<15	5	15	2005/2006
	0	7	20	2007
	6	2	5	2009
Pirimicarb	6	1	3	2005/2006
	1<R<3	1	3	2007
	no data	–	–	2009
Prosulfocarb	69	5	15	2005/2006
	32	2	5	2007
	27	2	5	2009
Pyraclostrobin	6	2	5	2005/2006
	117	2	5	2007
	8	1	3	2009
Pyrimethanil	19	5	15	2005/2006
	22	2	5	2007
	37	2	5	2009
Tau-fluvalinate	5<R<15	5	15	2005/2006
	20	5	15	2007
	10	5	15	2009
Tebuconazole	18	3	10	2005/2006
	260	5	15	2007
	10	5	15	2009
Tebufenozide	21	1	3	2005/2006
	108	1	3	2007
	31	1	3	2009
Tebufenpyrad	0	5	15	2005/2006
	91	5	15	2007

	0	1	3	2009
Terbuthylazine	109	1	3	2005/2006
	14	1	3	2007
	72	2	5	2009
Thiacloprid	199	1	3	2005/2006
	277	1	3	2007
	150	1	3	2009
Tolylfluanid	1178	5	15	2005/2006
	100	5	15	2007
	0	5	15	2009
Triadimenol	1<R<3	1	3	2005/2006
	0	5	15	2007
	0	1	3	2009
Trifloxystrobin	253	5	15	2005/2006
	0	4	12	2007
	8	2	5	2009
Vinclozolin	0	5	15	2005/2006
	23	5	15	2007
	13	5	15	2009

Taken from Thompson 2012:

Table 1.10: Residues in bee bread samples from colonies

Active ingredient	Application rate	Method	Residue µg/kg	RUD mg/kg	Reference
Captan	10 Ha Apples 2000 g ai/ha	10 colonies samples combined to single sample per day	4740 ± 1010 – 8090 ± 790	4.0	(Kubik et al., 2000)
carbaryl	Spray application to vines	Vines	0.5%		(Belliardo et al., 1975)
Carbaryl	Aerial spray application 1.1 kg/ha 65Ha	alfalfa	33-44	0.04	(Stanger and Winterlin, 1975)
chlorantraniprole	Soil application 253.5 g ai/ha	flowering Phacelia 2 tunnels	7 day <0.3		(Dinter et al., 2009)
chlorantraniprole	Spray application 60 g ai/ha	flowering Phacelia 2 tunnels	1 day 2863 7 days 108		(Dinter et al., 2009)
diazinon	Apples in flower 15L/ha	2 hives per site 2 sites	15- 16 days50- 90		(Skerl et al., 2009)
dichlofluanid	4.5 Ha, 5kg euparen /ha spray application	Strawberry 4 colonies	End of flowering 1.8 ± 0.5		(Kubik et al., 1992)
difenoconazole	10 Ha Apples 200 g ai/ha	10 colonies, samples combined to single sample per day	157 ± 67 – 411 ±75	2.1	(Kubik et al., 2000)
Flufenoxuron	Phacelia 40 g/ha	Tunnel -Germany	60	1.5	Flufenoxuron DAR
Flufenoxuron	Phacelia 40 g/ha	Field Spain	10-320	8.0	Flufenoxuron DAR
Flufenoxuron	Phacelia 40 g/ha	Field Italy	<0.01		Flufenoxuron DAR
Flufenoxuron	Phacelia 40 g/ha	Field France	10-70	1.8	Flufenoxuron DAR
Flufenoxuron	Phacelia 40 g/ha	Field France	20-120	3.0	Flufenoxuron DAR
Flufenoxuron	Phacelia 40 g/ha	Vineyard France	<0.01		Flufenoxuron DAR
Iprodione	4.5 ha in flower cherry plantation sprayed 0.75 kg ai/ha 6 days preflower and 0.188 kg ai/ha during flower	5 colonies sampling time during flowering	Mean 3055 ± 1436 (1795 ± 135 – 5511 ±2396)	29.3	(Kubik et al., 1999)
Methyl parathion microencapsulated	Birdsfoot trefoil treated at 1 kg ai/ha	2 nucleus colonies placed on edge of 0.5ha treated area every 48 hrs until 8 nuclei present, Identification of microcapsules in stored pollen	Mean 9.9% of pollen cells contaminated (max 15%)		(Burgett and Fisher, 1980)
Methyl parathion microencapsulated	Spray application 0.5 lb ai/acre (0.56 kg/ha)	alfalfa	9 days 530-1030 3.5 months 110-1030	2.1	(Johansen and Kious, 1978)

Active ingredient	Application rate	Method	Residue µg/kg	RUD mg/kg	Reference
			7.3 months 340-1170		
Methyl tiophanate	4.5 ha in flower cherry plantation sprayed 1 kg /ha 6 days preflower and 1 kg /ha during flower	5 colonies sampling time during flowering	Mean 1929 ± 1034 (196 ± 6 – 2904 ±75)	2.9	(Kubik et al., 1999)
monocrotophos	Aerial spray application 0.6 kg/ha 65Ha	alfalfa	28	0.05	(Stanger and Winterlin, 1975)
procymidone	4.5 Ha, 0.75 kg ai/ha spray application	Strawberry 4 colonies	End of flowering 1.2 ± 0.5	0.002	(Kubik et al., 1992)
Teflubenzuron	Oilseed rape semi-field study 157.5 g ai/ha	One colony 1 frame	1 day 23600 7 days 150	149.8	Teflubenzuron DAR
teflubenzuron	Oilseed rape field study 78.75 g ai/ha	One colony 2 frames	1 day 1710 4-14 days 110-160	21.7	Teflubenzuron DAR
thiacloprid	Apples in flower 96 g/ha	2 hives per site 2 sites	<LOD (10)		(Skerl et al., 2009)
Vinclozolin	4.5 ha in flower cherry plantation sprayed 375 g ai./ha	5 colonies sampling time during flowering	Mean 23628 ± 7045 (13107 ± 3754 – 31909 ± 7543)	85	(Kubik et al., 1999)

Combinations of pesticides detected in dead bees in the UK 1997-2011 (http://www.pesticides.gov.uk/guidance/industries/pesticides/topics/reducing-environmental-impact/wildlife/wildlife-incident-investigation-scheme.htm)

bumble bee	Boscalid; prothioconazole-desthio;tebuconazole
bumble bee	Chlorpyrifos; dieldrin
bumble bee	Azoxystrobin; boscalid; cypermethrin
honey bee	Myclobutanil; penconazole; pirimicarb; thiacloprid
honey bee	Carbendazim; dieldrin; HCH-gamma
honey bee	Bendiocarb; permethrin; propiconazole; tebuconazole
honey bee	Boscalid; carbendazim; *fluvalinate*; propiconazole; thiacloprid
honey bee	Chlorothalonil; cyproconazole; deltamethrin; *fluvalinate*
honey bee	Bendiocarb; deltamethrin; propiconazole
honey bee	Bendiocarb; DDE-pp; pirimiphos-methyl
honey bee	Dieldrin; HCH-gamma; permethrin; propiconazole; thiacloprid
honey bee	DDT-pp; fipronil; propiconazole
honey bee	DDT-pp; methomyl; propiconazole
honey bee	Chlorpyrifos; dimethoate; *fluvalinate*; thiacloprid
honey bee	Chlorpyrifos; cyhalothrin-lambda; difenoconazole; dimethoate; propiconazole; thiacloprid
honey bee	Chlorpyrifos; cyhalothrin-lambda; dimethoate; *fluvalinate*; thiacloprid
honey bee	Dieldrin; HCH-gamma; permethrin
honey bee	Chlorpyrifos; glyphosate; thiacloprid
honey bee	Permethrin; piperonyl butoxide
honey bee	MCPA; mecoprop-P
honey bee	Bendiocarb; imidacloprid; permethrin; tebuconazole

To conclude this Appendix, the conclusions and remarks as made by Thompson (2012) are given:

CONCLUSIONS & RECOMMENDATIONS

There are a variety of other routes of exposure where there is currently insufficient data to fully quanitfy their contribution to total exposure and further research is required:

If dusts are produced during sowing of treated seeds this may be a significant source of exposure and may result in residues in pollen and nectar of nearby flowering weeds or crops further work is required to develop robust methods to fully quantify this.

Inhalation may be a significant route of exposure for compounds with high vapour pressure and present in stored pollen or collected in water and further data are required.

Beeswax may be a significant route of exposure for highly lipophilic chemicals and more information is required to evaluate transfer to brood.

Water may be sourced from puddles or guttation droplets which may contain high residues for periods of days-weeks and further data is required on the relative importance of these routes.

There was insufficient data available to assess the exposure of bumble bees or solitary bee species. More data are required to fully evaluate the importance of differing routes of exposure for bumble bees and other non-Apis bees.

Other bees may be exposed to mixtures of pesticides through multiple applications, overspray of residues already present, e.g. systemic pesticides, collection of pollen and nectar from a variety of sources and stored within the nest. As previously there is a need to quantify this for non-Apis bees.

There is evidence in the literature of multiple residues of pesticides detected in honeybees, honey and pollen and wax within the hive but this is limited by the direction of the analysis to chemicals of interest to the researchers and rarely are levels of individual components reported. More data are required on realistic levels and combinations of pesticides at the individual colony level within the EU to more fully evaluate the effects of multiple pesticide exposure.

There are a large number of studies that have investigated the interactions between pesticides in honeybees. By far the majority have related to the interactions involving EBI fungicides and can be related to their inhibition of P450. The scale of the synergy is shown to be dose and season-dependent in acute exposures but there are few data relating to the effect of time between exposures or on chronic exposure effects at realistic exposure levels.

The vast majority of the studies have concentrated on the contact toxicity of the combinations. However the exposure section shows that a significant proportion of the exposure may be through ingestion of contaminated nectar. It appears that pesticides which induce P450s in other insects do not induce these enzymes in honeybees but natural chemicals, such as quercetin present in honey and propolis do induce P450s and reduce the toxicity of some pesticides. Given the role of the midgut enzymes in the metabolism of xenobiotics the shortage of data following oral exposure of mixtures is a major gap in our understanding of the potential interactions between chemicals, particularly those present in pollen and nectar, and the effects of diet quality in maintaining xenobiotic metabolising capacity within the gut.

Greater synergy is observed in the laboratory between EBI fungicides at field rates application rates and pyrethroids used as varroacides (flumethrin and fluvalinate) and between coumaphos and fluvalinate varroacides. Given the persistence of residues of varroacides detected in monitoring studies further evaluation of the combined effects of these with agricultural pesticides is warranted.

As effects are dose-dependent synergism between pesticides may be an area where modelling is applicable both from toxicokinetic/toxicodynamic and QSAR approaches but also needs to take into account formulation differences in affecting rate of uptake.

More recently data has shown that antibiotics used in hives may increase the susceptibility of bees to organophosphorus, pyrethroid and neonicotinoid insecticides through interaction with the membrane bound transporter proteins and further work is required to more fully understand the implications of these findings. It is therefore important that all treatments used on colonies used in studies are reported.

The exposure data demonstrate that bees are often exposed directly through applications of multiple active ingredients or indirectly through consumption of stored pollen and nectar to several pesticides over a period of time. Data are required to determine the effects of such long term low level exposure to multiple pesticides on the health and functioning of honeybee colonies foraging in agricultural environments.

There are data that may demonstrate increased spore counts of *N.ceranae* in bees previously chronically exposed to pesticides but there are also reports that spore count decreased following exposure to some pesticides. However, spore count may not be a reliable indicator of the impact of *N ceranae* infection in bees. There is a need for improved methods of assessment for some pathogens, e.g. *N cerenae* which more clearly link to the impact of the disease on the individual and the colony.

There are a wide range of factors which affect the immunocompetence of bees including the quality of the pollen diet, the presence of other diseases, such as *N ceranae*, or pests, e.g. Varroa, and in-hive treatments, such as antibiotics. In addition, the confinement of colonies or individuals may result in stress leading to immunosuppression. It is important that these factors are taken into account in studies determining the effects of pesticides on both individual and social immunity.

The effect of the diet on both the immunocompetence and the xenobiotic metabolising enzymes within the gut are important and impact on both the effects on the toxicity of other pesticides and the impacts on disease susceptibility. Pathogens may also impact on some measures of sublethal effects of pesticides. It is therefore important that the realistic routes of exposure are used in mixture studies, i.e. oral for contaminated pollen and nectar, and that the disease status of bees used in pesticide studies is fully understood.

Appendix IV: Draft experimental design WUR

Methoden:

Welke test: acuut contact (vooral in geval blootstelling direct door bespuiting), of acuut oraal (vooral bij blootstelling via voedsel, evt. indirect). Hier moet een keuze worden gemaakt, bij IWASA ging het om contact tox.

Insecticide:
Concentratie reeks om LD50 te bepalen: wij stellen voor een reeks van 7 concentraties: 10*LD50; LD50; 1/10*LD50; 1/100*LD50; 1/1000*LD50; 1/10.000*LD50; 0 (controle). We gaan tot 1/10.000 van de LD50 omdat in één geval een1100 keer hogere giftigheid werd gevonden.

Synergist:
Iwasa gebruikte de max concentratie die nog (net) geen verhoogde sterfte gaf. WUR zou er liever van uitgaan van de te verwachten (evt. maximale) velddosering, en daarnaast een nul (controle) en een 10 * velddosering, dus 3 series.

Welke bijen:
Iwasa gebruikte een mengsel van alle bijen van broedramen uit een paar volken, en gooide daar de (zichtbaar) jonge bijen uit (= tot een dag oud?). Als je bijen van een kantraam van het broednest neemt heb je minder jonkies. Wij zouden echter de voorkeur geven aan bijen van bekende leeftijd, ook omdat bekend is dat de gevoeligheid voor neonicotinoïden met de leeftijd behoorlijk kan verschillen. Wij zouden in dat geval willen kiezen voor bijen van 20 dagen of ouder: die zijn foerageerster. Dat is ten eerste de eerst en meest blootgestelde klasse, ten tweede zijn ze het meest gevoelig voor neonicotinoïden.

> Om dat te doen moeten in een aantal bijenvolken gedurende een paar dagen (bijna) alle die dag geboren bijen worden gemarkeerd (met een bepaalde kleur) en worden teruggezet in het volk. Voor de lab-proeven moeten ze dan op bijv dag 20 en op dag 24 na merken moeten worden teruggevangen om de proeven in te zetten (dan zou je meteen een vergelijking van twee leeftijden hebben). Werken met een bekende vaste leeftijd levert wel meer werk en dus kosten op.

NB EFSA Guidance suggereert om juist jonge bijen te gebruiken (of alleen bij de chronische tox?). In OECD en EPPO zitten vergelijkbare tegenstrijdigheden: jonge bijen; bijen van 'dezelfde leeftijd', en gewoon bijen van broedloze raat (= alle leeftijden?).

Uitvoering test:
Herhalingen minimaal 3 kooitjes met minimaal 10 (20, volgens CEB) per dosis. CEB eist 3 runs met 3 herhalingen.

Meenemen toxische standaard: 3 concentraties dimethoaat

Een eerst range finding is niet nodig, omdat LD50 bekend is en verwachte synergisme max rond factor 1000. Daarom steeds stappen van factor 10.

Een test voor 1 neo en 1 synergist:

		Neonicotinoïde						
		Concentratie * LD50						
synergist 1		10	1	0.1	0.01	0.001	0.0001	0
	0							
	veld							
	10*veld							

Dit geeft 21 combinaties: daaraan volgens EPPO en OECD nog toevoegen 3 concentraties van de positieve standaard: totaal 24 combi's Elke combi moet met minimaal 3 kooitjes met ieder minimaal 10 bijen (ik zou 20 voorkeur geven, zie ook CEB). Dus 72 kooitjes, 14140 bijen.