Betreft: Herbeoordeling risico voor bijen van middelen op basis van thiacloprid en acetamiprid – fase I B synergisme

Geachte mevrouw Dijksma,

Tijdens het AO bijensterfte op 16 mei 2013 heeft U de Tweede Kamer toegezegd het Ctgb te vragen thiacloprid en acetamiprid nationaal versneld te bezien met het oog op het risico voor bijen. Naar aanleiding van de afronding van fase I van het herbeoordelingsproject over de risico voor bijen van middelen op basis van thiacloprid en acetamiprid heeft het College, op 30 september 2013, uw ministerie, via de directeur van Plantaardige Agroketens en Voedselkwaliteit, gerapporteerd dat synergisme een potentiële rol speelt in de risicobeoordeling van bijen. Het Ctgb heeft geadviseerd dat in een vervolgfase een prioriteitenlijst van werkzame stoffen met synergistische interacties moet worden opgesteld voor alle werkzame stoffen. Hierbij rapporteer ik over de uitkomst van deze vervolgfase.

*Resultaat vervolgfase*
Om een accurate prioriteitenlijst van werkzame stoffen met synergistische interacties te kunnen opstellen is het noodzakelijk dat synergisme tussen stoffen kan worden voorspeld. Op basis van openbare literatuur heeft het College echter moeten concluderen dat er geen bestaande methodologie is waarmee het voorkomen van synergisme tussen stoffen accuraat kan worden voorspeld, zie bijlage I. Hierdoor kan een uitputtende prioriteitenlijst van stoffen voor synergisme niet worden opgesteld. Deze conclusie wordt ook bevestigd door recente publicaties van o.a. SCHER (SCHER/SCENIHR/SCCS, 2011) en specifiek voor bijen (FERA, 2012; EFSA, 2012).

Als gevolg van deze beperking heeft het College een inschatting gemaakt of acetamiprid of thiacloprid prioriteit heeft aangaande synergisme, en op een toekomstige prioriteitenlijst voor synergisme zou komen te staan. Het is aannemelijk dat acetamiprid of thiacloprid relatief hoge prioriteit krijgen omdat dit stoffen zijn die aantoonbaar betrokken zijn bij synergistische interacties voor bijen in laboratorium studies. De hoogste synergistische interacties zijn gevonden in een studie waar de bij werd blootgesteld aan zeer hoge concentratie fungiciden (Iwasa et al. 2004). Vanwege de hoge blootstellingconcentraties heeft het College geconcludeerd dat deze studie niet geschikt is voor een realistische extrapolatie van synergisme in veld relevante blootstelling van bijen. Synergisme is zeer afhankelijk van interne concentraties in de bij omdat stoffen andere cellulaire effecten kunnen veroorzaken op hogere interne concentraties dan bij lagere. In veldstudies is gebleken dat voor thiacloprid het synergistisch effect verwaarloosbaar is met verscheidene EBI-fungiciden (Schmuck et al. 2003). Resultaten van beschikbare veldstudies van andere stoffen laten eenzelfde beeld zien dat gevonden interacties bij hoge concentraties in het laboratorium niet in het veld worden waargenomen. Synergistische interacties bij hoge blootstellingsconcentraties in het laboratorium kunnen dus niet rechtstreeks worden toegepast in de risico analyse voor bijen in de veldsituatie. Studies waar dit wel worden gedaan, zoals de studie van Sanchez-Bayo and Goka (2014), worden daarom niet als relevant geacht in het huidige herbeoordelingsproject.

Op basis van studies waar veldrelevante concentraties zijn getest op synergisme heeft het College geconcludeerd dat er geen aanleiding is om de middelen op basis van acetamiprid en thiacloprid versneld nationaal te herbeoordelen.

*Aanbevelingen voor vervolg*
De onderstaande aanbevelingen betreft aanbevelingen voor zowel het Europese en nationale speelveld.

Op lange termijn dient de kaders te worden opgesteld en de methodieken ontwikkeld in het Europese speelveld om synergisme bij de stof- en middelbeoordeling te betrekken. Op de kortere termijn is enkel mogelijk een lijst op basis van geïdentificeerde combinaties uit openbare literatuur op te stellen, en hiervan een prioriteitenlijst maken. Deze lijst zal niet uitputtend zijn. EFSA is in Europa verantwoordelijk voor methodiekontwikkeling en stofbeoordeling. Ctgb zal dan ook EFSA vragen hieraan uitvoering te geven. Het College zal betrokken blijven bij verdere vervolgstappen.
Daarnaast wordt in Europees verband de stof acetamiprid en thiacloprid herbeoordeeld. Nederland is beoordelend lidstaat van acetamiprid in Europees verband en het College zal de beoordeling van thiacloprid nauwlettend volgen.

Commented [51.2e]: Een term. Ctgb of College

Bijen foerageren op een groot gebied en zijn blootgesteld aan vele stoffen, inclusief gewasbeschermingsmiddelen, maar ook aan diergeneesmiddelen en andere organische en anorganische verontreinigingen. Al deze stoffen kunnen zich ophopen in producten zoals bijenwas en honing. De som van de toxiciteit van al deze stoffen, mengseltoxiciteit, wordt voor een beperkte mate bepaald door synergisme. Voor ecologisch relevante effecten, zoals sterfte en reproductie, wordt er slechts in 5 tot 10% van de onderzochte stofcombinaties een mengselinteractie gevonden. In 90 tot 95% van de gevallen is het risico van twee stoffen goed te voorspellen op basis van hun individuele effect, zonder interacties. Indien er een interactie optreedt, is de grootte van een synergistische interacties waarschijnlijk dermate laag bij veldrelevante blootstellingsconcentraties dat synergisme maar een relatief klein deel bijdraagt aan de gehele mengseltoxiciteit die een bij ondervindt. Omdat de stoffen worden beoordeeld in verschillende kaders, adviseert het College u daarom op nationaal niveau een overkoepelende mengseltoxiciteit beoordeling voor de verschillende stoffen in bijenwas, honing en pollen uit te laten voeren in gebieden waar een hoge bijensterfte wordt geobserveerd door gespecialiseerde kennisinstituten (zoals het RIKILT, RIVM) in samenwerking met het Ctgb.

Hoogachtend,
De voorzitter van het College voor de toelating van gewasbeschermingsmiddelen en biociden,

Ir. J.F. de Leeuw

Referenties

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**Bijlage I Herbeoordeling van risico voor bijen van thiacloprid en acetamiprid**
**fase I B: synergisme in risicobeoordeling.**

## Introduction

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). Besides the above mentioned plant protection products (ppp) (honey) bees may encounter a multitude of other stressors while foraging for food. Since bees are important pollinators of both managed crops and wild flora and 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. 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 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 ppp 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.

In order to review the necessity of a possible re-assessment of the two neonicotioids acetamiprid and thiacloprid CTGB first recognized that the over a 100-fold increase in toxicity of thiacloprid and acetamiprid when combined with piperonyl butoxide, triflumizole and propiconazole should be assessed in light of other possible potential pesticide combinations which have synergistic interactions. 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

Thirty-eight active ingredients 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. The majority was also found to be presence in bee products like honey, bee bread, bee wax and dead bees (o.a. Yáñez et al. 2013; Pohorecka et al. 2012; Mullin et al. 2010; Thompson, H.M. 2012). At the time of the literature search 18 of these active ingredients were authorised in one or more ppp

in the Netherlands. 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'. Only a few semi-field studies are available, executed for a limited amount of active ingredients including acetamiprid and thiacloprid. Unfortunately not the exact combinations that triggered the immense synergistic effects described by Iwasa et al. (2004) in laboratory-studies were tested. However, in combination with other EBI-fungicides (o.a. tebuconazole), no increased toxicity was detected. 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 available 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 I, 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 Table 1 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 I). 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.

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<br>fungicide chlorothalonil | (Thompson and Wilkins 2003) |

**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 way forward**

- 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 EFSA will be asked to**

1. Determine the ground works on which synergism can be included into the risk assessment. In addition, develop the methodologies needed to incorporate mixture toxicity into risk assessment.
2. Until such a new methodology is established, EFSA is asked to construct a list of all known synergistic combinations from (public) literature and set data requirements for applicants to address the synergistic potential.
3. Build a database with all available information concerning an active substance (persistence, dispersal, ecotoxicogical data, MoA, known interactions, etc.) 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 EFSA**

4. 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).
5. Assess if alternatives, like the development of screening assays for interactive potential, should also be included in future prioritizations of substances for synergistic interactions.

**National developments**

6. CTGB will co-operate on a national level with specialised knowledge institutes (like RIKILT, RIVM) to develop a cumulative risk assessment for honey bees from exposure to various chemicals through multiple exposure sources (like pollen, honey) in areas that suffer from high unexplained mortalities.

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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 demonstrated 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. 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 the assumption that synergistic interactions in the field are much lower than in laboratory studies 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.
EFSA (2012) also recommends to conduct toxicological studies in adult bees and bee larvae using realistic application concentrations to determine the threshold of toxicity and the magnitude of the synergism. They advise that the design of ecotoxicological mixture studies of potential synergists should take into account the toxicokinetics of the synergist (half life) and the dose dependency of the synergy. Consequently a full dose response can be generated to determine the magnitude of the interaction at concentrations of environmental relevance, and both the maximum potentiating factor of the synergist and the concentration of which no potentiating factor may occur in the dose response curve. In general, overall, when sufficient studies have been conducted, a number of options would be available to the risk assessor and

can be applied in future risk assessment i.e. to derive species-species, chemical-specific, mixture specific or class-specific adjustment factors using the full dose responses, benchmark doses, their limits or species sensitivity distributions and the magnitude of the interaction (EFSA, 2012).

**Conclusion of 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 EFSA.

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## Appendix I:

**Proposal: 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 toxicokinetic –phase (or both). However, reviews on pesticide mixture data revealed that most mixture effects are accurately described by the concept of concentration addition. Sever 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 methods are 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.

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

**Main omissions 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 of effects, 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, it 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 alternating. 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 in 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 theoretically 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, for ppp's, several authors have assessed the accuracy of CA to predict mixture toxicity: (cited by Van Gestel *et al.* 2011*)."Deneer (2000) reviewed the usefulness of CA for describing combination effects of pesticides on aquatic organisms. Pesticides are extremely well characterised in terms of mode of action and are therefore ideal as reference cases to study the relevance of mechanistic information for anticipating joint toxicity. 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 abovementioned 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.
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 even more, it has been recognized that different endpoints may show 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, not all life stages might be equally sensitive (and susceptible for that matter). The exposure duration may also influence mixture effects, as it is widely known that effect concentrations are exposure time 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 interaction.

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, the route(s), timing, life stage, endpoint assessed and duration of exposure and 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 an 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 I-I 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 known. Also for other field methodologies to screen substances for their MoA have been developed, like path-way toxicity endpoints 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 addressed before the findings from high-throughput screens and *in silico* models may be considered sufficiently robust and informative' (Rusyn and Daston, 2010).

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."*

To complicate matters more, 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 grouping of chemicals into groups with (presumed) 'similar' MoA has been issued (o.a. ECHA, OECD, ECHA, EPA) although without guidance. There is currently no general agreement on the scientifically best approach and 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 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). Following 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 into the ecological risk assessment (ERA) by (assuming some sort of) CA of the individual a.i.'s, based on only the endpoint of interest. 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 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 sized to account for mixture effects (Backhaus et al. 2013, Kortenkamp et al. 2009).

Institutes like EFSA, 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, 2000a, 2000b, 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. US EPA have 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 can not be used to determined potential synergisms of the mixture components. It merely determines the (concentration )risk bar for cumulative exposure of a group of similar acting chemicals.

Conclusion
Mixture effect should be included in ERA, but it is currently not feasible to include it in the ERA of individual plant protection products! Although lacking robust data, the reference model CA seems to accurately describe most tested mixtures, including the use of this model is a very good start.

***Assessing potential additive risks from PPPs***
Assessing potential additive risk from ppp's 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 ppp's in water, soil and other exposure media., and help to further priorities which mixture (in effect concentrations) or mostly encountered.
However, more information can and should be assessed from (standardized) toxicity tests. Development of toxicity in time, multiple endpoints etc could be recorded, shifting from default to a more detailed behavioural pattern of the chemical under investigation, as well as the test organism. This was illustrated in Figure 1 for human risk assessment by Meek (2009).

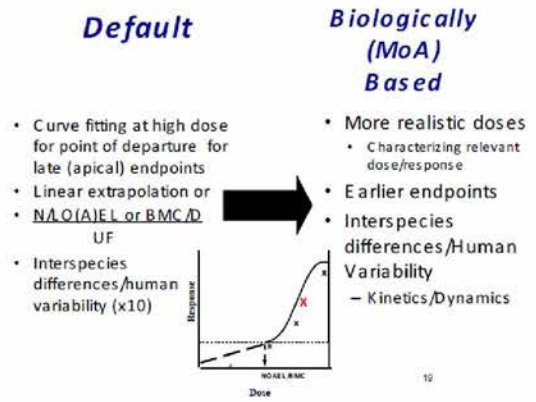


**Figure 1.** Moving from Default to More Mode of Action Based Approaches in Chemical Risk Assessment

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 solely synergism in the risk assessment of individual PPPs is insufficient if mixture toxicity itself is not fully addressed.

***PPP regulation***

The current PPP regulation does not give legal basis to address mixture toxicity for the ERA of individual PPPs. Mixture toxicity is currently limited to the presence of multiple active ingredients (a.i.'s.) in one ppp formulation or tank mixture.

***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. Sever 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 methods are 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.

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

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**Appendix I-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<br>fungicide chlorothalonil | (Thompson and Wilkins 2003) |

Especially with the known P450 inhibiting substances, the presumed synergistic MoA is believed to take place in inhibiting 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).