SCIENTIFIC OPINION

Scientific Opinion on the science behind the development of a risk assessment of Plant Protection Products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees)[1]

EFSA Panel on Plant Protection Products and their Residues (PPR) [2, 3]

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ABSTRACT

The PPR Panel was asked to deliver a scientific opinion on the science behind the development of a risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). Specific protection goals options were suggested based on the ecosystem services approach. The different routes of exposure were analysed in detail for different categories of bees. The existing test guidelines were evaluated and suggestions for improvement and further research needs were listed. A simple prioritisation tool to assess cumulative effects of single pesticides using mortality data is suggested. Effects from repeated and simultanous exposure and synergism are discussed. Proposals for separate risk assessment schemes, one for honey bees and one for bumble bees and solitary bees, were developed.

KEY WORDS

Guidance Document, PPR opinion, honey bees, bumble bees, solitary bees, pesticide, risk assessment

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SUMMARY

Following a request from the European Commission, the Scientific Panel on Plant Protection Products and their Residues (PPR Panel) of EFSA was asked to deliver a scientific opinion on the science behind the development of a risk assessment of Plant Protection Products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). The opinion will be the scientific basis for the development of a Guidance Document which should provide guidance for notifiers and authorities in the context of the review of Plant Protection Products (PPPs) and their active substances under Regulation (EC) 1107/2009.

For the development of robust and efficient environmental risk assessment procedures, it is crucial to know what to protect, where to protect it and over what time period. Specific protection goals based on ecosystem services were suggested according to the methodology outlined in the Scientific Opinion of EFSA (2010). Pollination, hive products (for honey-bees only) and biodiversity (specifically addressed under genetic resources and cultural services) were identified as relevant ecosystem services. It was suggested to define the attributes to protect as survival and development of colonies and effects on larvae and honey bee behaviour as listed in regulation (EC) No 1107/2009. In addition, abundance/biomass and reproduction were also suggested because of their importance for the development and long-term survival of colonies. The magnitude of effects was defined as negligible if the natural background mortality compared to controls was not exceeded. Further work is needed to give recommendations on the deviation from the controls up to which an effect is still considered negligible. The current methods of field testing would need major improvements in order to detect for example an increase in daily mortality of foragers by 10% with high statistical power. Based on expert judgement it was considered that a small effect could be tolerated for few days without putting the survival of a hive at risk. Further research (modelling) is proposed to clarify this question and to revise the proposal for the magnitude of effects and the temporal scale of effects. The current risk assessment for honey bees relies on an Hazard Quotient (HQ) approach (application rate/LD50) in lower tiers and on semi-field and field tests in higher tiers. It is particularly difficult to ascertain whether a specific exposure percentile is achieved in field studies. Decisions need to be taken on how conservative the exposure estimate should be and what percentage of exposure situations should be covered in the risk assessment. It is recommended to design a flow chart for checking whether exposure in the semi-field or field study was indeed higher than that corresponding to the desired percentile. Factors that may be included are: the crop and its developmental stage, the dosage, measures ensuring that bees are coming into contact with the compound/formulation, weather conditions, and for instance the generation of guttation droplets by the crop. The final decision on protection goals needs to be taken by risk managers. There is a trade-off between plant protection and the protection of bees. The effects on pollinators need to be weighted against increase in crop yields due to better protection of crops against pests.

Residues in different environmental matrices and bee products were combined with estimates of exposure of different categories of bees. Highest concentrations of residues were found after spray treatments in pollen and nectar. Residues in guttation droplets showed a wide variability due to the number of parameters known to influence guttation production (environmental conditions, crop type, growth stage, etc.). A potentially high exposure was highlighted for bees in some crops (e.g. maize). Exposure to dust drift from sowing treated seeds was identified as a relevant exposure route. The exposure of different categories of bees from different sources and for different application techniques suggests that the potential risk from oral uptake was highest for forager bees, winter bees and larvae. The exposure of nurse bees occurs via a combination of pollen and nectar, of larvae by contact to wax and foragers, drones, queens and swarms intercepting droplets and vapour by contact and inhalation.

Worker bees, queens and larvae of bumble bees and adult females and larvae of solitary bees were considered to be the categories that are most exposed via oral uptake. Larvae of solitary bees consume large mass provisions with unprocessed pollen thus, compared with honey bee larvae, they are more exposed to residues in pollen. Moreover, bumble bees and solitary bees may be exposed to a larger

extent via contact with nesting material (soil or plants) compared to honey bees, suggesting the need for a separate risk assessment for bumble bees and solitary bees.

For the ranking of bees, the inclusion of multiple exposures with appropriate weights would need to be done with a modelling or scenario-based approach that was not available in the current assessment. It was therefore recommended that the categories of bees which represent the worst-case exposure scenarios through multiple exposures are further assessed (e.g. honey bee nurses) and that those categories which highlighted potential but unknown exposures through consumption of water and inhalation of vapour in/out field are further analysed with more studies. Further research is recommended on the testing of the presence and fate of residues (e.g. in bee relevant matrices and in-hive following spray and dust applications) and on the development of reliable exposure models.

The overview of the available studies on sub-lethal doses and long-term effects of pesticides on bees highlighted gaps in knowledge and research needs in the following areas: more toxicological studies to be performed in bees for a wider range of pesticides on both adults and larvae including sub-lethal endpoints, also including contact and inhalation routes of exposure. Few studies were conducted with non-*Apis* bees, considering endpoints such as fecundity (e.g. drones production in *Bombus* and cell production rate in solitary bees), larvae mortality rate, adult longevity and foraging behaviour. The use of micro-colonies in bumble bees appears to be well-suited to measure lethal and sub-lethal effects of pesticides with low doses and long-term effects.
Because of the specific toxicokinetic profile of bees compared with other insects, it is recognised that toxicokinetic data can provide useful information on the potential biological persistence of a pesticide which, in some cases, could have effects after continuous exposure that maybe more marked compared with their short-term effects. The integration of toxicokinetic knowledge and low (sub-lethal) dose effects generated from laboratory and field studies in the hazard identification and hazard characterisation of pesticides in *Apis* and *non-Apis* bees can provide a better understanding of short-term and long-term effects. It is therefore concluded that the conventional regulatory tests based on acute toxicity (48 to 96 h) are likely to be unsuited to assess the risks of long-term exposures to pesticides.
A testing protocol and mathematic model, based on Haber's law, have been developed as a simple prioritisation tool to investigate the potential effects after repeated exposure to single pesticides using mortality data. However, a number of assumptions inherent to the model raise uncertainties. The protocol and model needs further validation in the laboratory and to be tested for sub-lethal endpoints in adult and bee larvae. Finally, combining basic toxicokinetic data for an active substance and its metabolites, such as the half life, will also provide more precise estimates on the potential of bioaccumulation. In the case of potential persistence of the active ingredient, half life of the parent compound and its metabolites should be determined in larvae, newly emerged bees and foragers.

The working group identified the need for improvement of existing laboratory, semi-field and field testing and areas for further research. Several exposure routes of pesticides are not evaluated in laboratory conditions, such as the intermittent and prolonged exposures of adult bees, exposure through inhalation and the exposure of larvae. Likewise, the effects of sub-lethal doses of pesticides are not fully covered in the conventional standard tests.

Sub-lethal effects should be taken into account and observed in laboratory studies. Potential laboratory methods to investigate sub-lethal effects would be testing of *Bombus* microcolonies to investigate effects on reproduction, proboscis extension reflex (PER) test for neurotoxic effects and homing behaviour for effects on foraging, including orientation. Further research is needed in order to integrate the results of these studies in the risk assessment scheme.

Semi-field testing appears to be a useful option of higher tier testing. Nevertheless, weaknesses have been identified for each of the test guidelines e.g. the limited size of crop area, the impossibility to evaluate all the possible exposure routes of the systemic compounds used as seed- and soil-treatments (SSST), the limited potential to extrapolate the findings on larger colony sizes used in field studies or the relatively short timescale (one brood cycle).

The guideline for field testing (EPPO 170) (4) has several major weaknesses (e.g. the small size of the colonies, the very small distance between the hives and the treated field, the very low surface of the test field), leading to uncertainties concerning the real exposures of the honey bees. The guideline is better suited to the assessment of spray products than of seed- and soil-treatments. Points for research and improvement of methods used in field testing are highlighted (e.g. methods for detection of mortality).

The available protocols for testing of solitary bees are suitable to study the oral and contact toxicity in adults and larvae for several species of solitary bees (*Megachile rotundata*, *Osmia* spp.) but they need to be ring tested. More studies are necessary to compare the susceptibility of honey bees with other non-*Apis* species in order to see to which extent honey bee endpoints also cover non-*Apis* bees.

Future research is recommended to improve laboratory, semi-field and field tests (e.g. extrapolation of the endpoints in first tier to the colony/forager effects, extrapolation of the toxicity between dust and spray, extrapolation of laboratory based *Bombus* micro colonies to *Apis* and solitary bees).

Pesticides are often applied in tank mixes (2 to 9 active ingredients at the same time) and in addition non target organisms will be exposed to mixtures of compounds following sequential applications to crops. There is a consensus in the field of mixture toxicology that the customary chemical-by-chemical approach to risk assessment is too simplistic. At low levels of exposure concentration, addition has been observed more often than synergistic or antagonistic effects for mixtures of pesticides with a common mode of action and independent action (response addition) has been observed for compounds with a different mode of action. In some cases synergistic and antagonistic effects have also been observed.

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 the case of synergism which can be predicted based on the mode of action of the chemical classes involved (e.g. EBI fungicides and insecticides), and in the absence of existing data on toxicity of the mixture, it is recommended to design full dose-response studies in adult bees and larvae for mixtures of potential synergists. Further work is also required to identify the molecular basis of interactions between environmentally realistic exposure to pesticides and the range of honey bee diseases (fungi, bacteria and viruses) to determine whether and how these may be included in risk assessment.

Separate risk assessment schemes are proposed, one for honey bees and one for bumble bees and solitary bees. In the first tier it is suggested to include toxicity testing that covers a longer period of exposure (7 to 10 days) for adult bees as well as larval bees. Both life stages can be exposed for more than one day and this risk was not covered by the standard OECD tests (213 and 214) for oral and contact exposure. Currently there is insufficient evidence that toxicity following extended exposures can be reliably predicted from acute oral LD50 data. It is also proposed to investigate whether there are any indications of cumulative effects for each compound. A new method to detect cumulative toxicity is proposed based on Haber`s law. If there is an indication that a compound is a cumulative toxin then this needs further evaluation since the potential effects of prolonged or repeated exposure to low doses may be underestimated.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

EFSA is currently revising the European Guidance Document on terrestrial ecotoxicology elaborated by the Commission and experts from Member States. In the context of this revision, the bees risk assessment will also be addressed.

Member of the European Parliament and beekeepers associations have expressed their concerns towards the Commission as to the appropriateness of the current risk assessment scheme, and in particular on the EPPO[4] "Environmental risk assessment scheme for plant protection products – Chapter 10: honeybees" revised in September 2010 with ICPBR[5] recommendations.

Considering the importance and the sensitiveness of this issue, and in line with the aim of the Commission Communication on Honeybee Health (COM(2010) 714 final)[6] adopted on 6 December 2010, the Commission considers that the revised EPPO assessment scheme would need further consideration by EFSA in an opinion on the science behind the risk assessment for bees and that a Guidance document on the risk assessment of Plant Protection Products on bees should be developed.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

A scientific Opinion of the PPR Panel on the science behind the development of a risk assessment of Plant Protection Products on bees (*Apis mellifera*, *Bombus spp*. and solitary bees) will be prepared.

In particular the following issues will be addressed:

- The assessment of the acute and chronic effects of Plant Protection Products on bees, including the colony survival and development.
- The estimation of the long-term effects due to exposure to low concentrations
- The development of a methodology to take into account cumulative and synergistic effects.
- The evaluation of the existing validated test protocols and the possible need to develop new protocols, especially to take into account the exposure of bees to pesticides through nectar and pollen.

In order to have the possibility for stakeholders and the interested public to comment on the draft Guidance Document, we propose to include a round of public consultations on the draft Guidance Document. An Opinion on the science behind the Guidance Document could be delivered by April 2012 and a final Guidance Document in December 2012.

[4] European and Mediterranean Plant Protection Organization
[5] International Commission for Plant-Bee Relationships Statutes
[6] Communication from the Commission to the European Parliament and the Council on Honeybee Health, COM(2010) 714 final, adopted on 06/12/2010

1 CHAPTER 1: INTRODUCTION

A decline of some pollinator species was reported in several different regions of the world (Biesmeijer et al., 2006; Committee on the status of Pollinators in North America, 2007). Bee poisoning incidents were reported in Europe (e.g. exposure to dust from seed treatments). Pollination is a very important ecosystem service for food production and maintainance of biodiversity (Gallai et al., 2009). The question on the causes of the observed declines received a lot of attention from regulatory authorities. Research activities and montoring of honey bee colony losses and bee poisoning incidents were initiated.

Pesticides were often considered as one factor among others to contribute to the decline of some insect pollinator species. Concerns were were raised by Members of the European Parliament and beekeepers' associations on the appropriateness of the current risk assessment schemes for plant protection products. The European Commission tasked EFSA to issue an opinion on the science behind the risk assessement for bees and to develop a Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp., and solitary bees).

Health status of bees in relation to multiple stressors is an important issue under discussion. The bee health aspects are dealt with under the animal health legislation. The current opinion is targeted to the pesticides regulation.

The final Guidance Document is intended to provide guidance for notifiers and authorities in the context of the review of Plant Protection Products (PPPs) and their active substances under Regulation (EC) 1107/2009. The current scientific opinion will provide the scientific basis for the development of the Guidance Document. A public consultation is foreseen in order to give stakeholders and the interested public the opportunity to comment on the draft Guidance Document.

The current opinion and the subsequent Guidance Document are focused on the risk assessment for bees (honey bees, bumble bees and solitary bees). The risk to other pollinating insects (e.g. belonging to Lepidoptera and Diptera) is outside the scope of this opinion. The risk to other insect pollinators would need to be addressed in the risk assessment for other non-target arthropods.

The process of the development of the guidance document follows the methodology of definition of specific protection goals as outlined in the Scientific Opinion of EFSA's PPR Panel (EFSA 2010). Risk management choices need to be made to define the specific protection goals. The Standing Committee on the Food Chain and Animal Health will be consulted for the appropriate levels of protection (e.g. to make choices on the magnitude of effects, duration of effects and exposure percentiles). Specific Protection Goal options for the dialogue with Risk Managers are proposed in this opinion.

A tiered risk assessment scheme is outlined in this scientific opinion and will be further developed based on the final agreed specific protection goals, with a simple and cost effective first tier to usually more complex higher tier studies under semi-field and field conditions. Each of the tiers will have to ensure that the appropriate level of protection is achieved.

Relevant literature known by the experts was incorporated in the current opinion. Given the limited timeframe it was not possible to conduct full systematic literature reviews. Therefore, the PPR panel is not able to guarantee that all published information was incorporated in the current opinion.

2 CHAPTER 2 SPECIFIC PROTECTION GOALS

2.1 Specific protection goals in the context of pesticides regulation and ecosystem services

For the development of robust and efficient environmental risk assessment procedures it is crucial to know what to protect, where to protect it and over what time period. Regulation (EC) No 1107/2009 concerning the placing of plant protection products on the market broadly describes general protection goals under Chapter II, Article 4.3 (complementary criteria for the residues of pesticides are in Article 4.2):

"A plant protection product, consequent on application consistent with good plant protection practice and having regard to realistic conditions of use, shall meet the following requirements: *[....]*

(e) it shall have no unacceptable effects on the environment, having particular regard to the following considerations where the scientific methods accepted by the Authority to assess such effects are available:

(i) its fate and distribution in the environment, particularly contamination of surface waters, including estuarine and coastal waters, groundwater, air and soil taking into account locations distant from its use following long-range environmental transportation;

(ii) its impact on non-target species, including on the ongoing behaviour of those species;

(iii) its impact on biodiversity and the ecosystem"

Honey bees are specifically considered under point 3.8.3 of the Annex II of the regulation:

"An active substance, safener or synergist shall be approved only if it is established following an appropriate risk assessment on the basis of Community or internationally agreed test guidelines, that the use under the proposed conditions of use of plant protection products containing this active substance, safener or synergist:

— will result in a negligible exposure of honeybees, or

— has no unacceptable acute or chronic effects on colony survival and development, taking into account effects on honeybee larvae and honeybee behaviour."

The general protection goals of the EU regulation need to be defined clearly for the development of an efficient risk assessment scheme. The methodology of definition of specific protection goals follows the approach outlined in the Scientific Opinion of EFSA (2010).

The working group identified pollination, biodiversity and hive products (for honey-bees only) as relevant ecosystem services. Besides food/feed (e.g. honey, pollen, larvae in some countries, wax for food processing, propolis in food technology, royal jelly as a dietary supplement and ingredient in food), honey bees' products are also used as natural medicines (e.g. honey as an ingredient in medicine-like products, pollen, wax as a coating agent, propolis, royal jelly), cosmetic (e.g. pollen, wax, propolis, royal jelly), preservatives (e.g. for the tobacco industry), treating agents (e.g. for meat packing and coating coffee), textiles (e.g. beeswax is used to waterproof textiles and papers; emulsions containing beeswax for leather treatment). A more complete overview on bee products and their use can be found in Krell (1996).

Biodiversity itself is not listed as an ecosystem service in the Scientific Opinion of EFSA (2010) but biodiversity is often seen as the provider of ecosystem services. In the EFSA opinion biodiversity is specifically addressed under the ecosystem services "genetic resources" and "cultural services" such as education, recreation and aesthetic values.

Many agricultural crops depend on pollination. About 70% of the main crops used directly for human consumption in the world are insect pollinated (Klein et al. 2007). The economic value for the contribution of pollinators to the production of crops used directly for human consumption (excluding the value of non-food agricultural production, cattle raising and natural vegetation) was estimated as € 153 billion, which is about 9.5% of the total value of the production of human food worldwide (Gallai et al., 2009).

There is a trade-off between plant protection and protecting the ecosystem services, pollination, hive products and biodiversity. From a farmer's point of view plant protection may be more important than hive products. While for beekeepers, hive products are of greater importance. Society may give a high value to protection of biodiversity (to ensure delivery of other ecosystem services such as aesthetic values, cultural services and genetic resources).

In order to take account of possible trade offs it is proposed to set different protection goals for in-field and the off-field areas. For example less conservative protection goals could be set in-field than off-field.

Pollination service and biodiversity are linked. A parallel decline of pollinators and insect pollinated plants were observed in Britain and the Netherlands (Biesmeijer et al., 2006). Impacts on pollinator populations may reduce plant biodiversity because of effects on reproduction of insect pollinated plants.

For pollination service no effects threshold can be given which should be not be exceeded. Linear relationships were observed between crop yields and density of pollinators, e.g. in blueberries (Dedej and Delaplane, 2003), oilseed rape (Steffan-Dewenter, 2003), seed yields of flowering plants increased with abundance of flies (Clement et al., 2007).

An optimal number of honey bee hives per ha and the number of nesting females of solitary bees can be found in literature for certain crops (see Appendix A). These figures give a rough estimate on the number of honey bee colonies and solitary bees that are required for an optimal crop yield. The specific protection goal for abundance of honey bees and solitary bees could theoretically be based on these figures – e.g. the application of a pesticide should not decrease the number of nesting females of solitary bees below these thresholds. However in reality the number of nesting solitary bees and the number of bee hives will vary greatly and therefore it would be very difficult to use these numbers directly in the risk assessment. As a surrogate, effects defined as percentage of mortality of bees are suggested.

The risk assessment scheme (see Chapter 6) and the test methods (see Chapter 5) need to ensure that the specific protection goals can be assessed. For example, test methods need to be able to detect a certain magnitude of effect as defined in the specific protection goal and the risk assessment scheme needs to be constructed in such a way that all attributes suggested in the specific protection goals are covered at the appropriate level of exposure.

2.2 Definitions of the dimensions of the Specific Protection Goals (SPGs)

2.2.1 Ecological entities

In the Specific Protection Goals (SPG) approach, the PPR Panel proposed a range of points for the dimension "ecological entity": i.e. the "individual", the "(meta) population", the "functional group" and the "ecosystem" (EFSA, 2010; Nienstedt et al., 2011).

For honey bees above the "individual" ecological entity, there is the "colony" which is neither a "population" nor a "metapopulation" as defined by EFSA (2010)[7].

To understand how honey bees fit into the SPG scheme (i.e. at which ecological entity they should be considered), the following clarifications are given:

A **honey bee colony** is perennial and consists of different **categories (or subgroups) of bees** of different castes, sexes and ages (i.e. the non-reproductive females = the workers, the reproductive females = the queens, the males = the drones; the larvae and the adult workers). Depending on age, worker bees achieve different tasks like nursing, attending the brood, cleaning the cells, storing food, producing wax, constructing combs, guarding and foraging (see Robinson, 1992).

A **honey bee population** is a group of colonies which is spatially defined by the mating area, i.e. the area covered by drones and queens to mate. At the mating area, sometimes also known as drone congregation area (Ruttner and Ruttner, 1966, 1972; Taylor and Rowell, 1987), the genotyping of drones showed that the size of a population of honey bees is about 240 colonies (Baudry et al., 1998).

A **honey bee metapopulation** is a set of multiple honey bee populations. Currently, almost 7000 genotyped colonies were found to belong to this metapopulation (see Rortais et al., 2011).

In honey bee colonies, the queen is unable to live alone (e.g. she does not forage), nor do workers form viable colonies (e.g. they cannot mate and therefore cannot produce female offspring). The various tasks fulfilled by the different categories of bees are all important for the functioning of the colony as a whole. In that sense, honey bee colonies are referred to as "super organisms" (Moritz and Southwick, 1992), a variant of the "organism" concept according to Pepper and Herron (2008). The "organism" can also be referred to an "individual organism" (Ghiselin 2011).

In order to protect honey bees, the colony as a whole needs to be protected (i.e. the various categories or subgroups of honey bees) as well as its various members (i.e. the individual honey bees). Therefore, it is proposed to consider the colony as an ecological entity placed between the "individual" and "population". The colony would contain the various "categories/subgroups of honey bees".

For social non-*Apis* bees the same ecological entity as for honey bees may be appropriate (that is, to consider the colony as a super organism) even if the queens of bumble bees are able to live alone, at least in part of their life cycle.

In solitary bees, each female builds and provisions her own nest without assistance from other bees, in this case the definition of population given for other animals is applicable (see Appendix C). A population is a group of interbreeding individuals of the same species inhabiting the same area and isolated from other groups. A population of solitary bees can be spatially defined by its habitat size. Most solitary bees are gregarious in that numbers of individuals nest in close proximity to each other. Mating occurs near the emerging place and females tend to nest at or in proximity to the natal nesting site. Nesting females of solitary bees are central place foragers, which means that each female has to return to its nest several times a day for nest building and brood provisioning. The homing ability of the pollinator can provide the maximum foraging range and should also provide an estimate of its potential habitat size. Foraging range is probably less reliable in estimating habitat size, as it is dependent on multiple factors, such as distribution of resources (Schneider and McNally, 1993), availability of the resources during the season, or structure of the landscape (Steffan-Dewenter and

[7] In EFSA (2010), p33: a metapopulation is defined as a "population of populations" of the same species connected through immigration and emigration (Hanski and Gyllenberg, 1993).

Kuhn, 2003). For example, the homing distance of *Osmia cornuta* is 1800 m, but with abundant floral resources (peak orchard bloom), most females forage within 100 – 200 m of their nests (Vicens and Bosch, 2000). In *Osmia lignaria*, the homing ability has been established at 1.2 km (Guédot et al., 2009) but its foraging area is within 400 m. Zurbuchen et al. (2010) showed that two other solitary bees ,*Chelostoma florisomne* and *Hoplitis adunca,* are able to cover a distance of up to 480 and 650 m, respectively, to reach the flowering resources and the investigated landscape structures (such as forests, hills, rivers and motorways) did not act as insuperable barriers. Compared with honey bees and bumble bees, solitary bees show much smaller maximum foraging ranges (see Table 1 in Zuburchen et al., 2010).

A metapopulation as defined by EFSA (2010) is a "population of populations" of the same species connected through immigration and emigration (Hanski and Gyllenberg, 1993). Currently, the habitat of many species is fragmented, resulting in small local populations with individuals occasionally dispersing between the remaining habitat patches. Franzen and Nilsson (2010) showed that metapopulation survival in solitary bees is linked with the population size and the pollen resources availability at landscape level. Many species of solitary bees show a wide distribution range and are exposed to diverse climatic conditions, and thus exhibit substantial variation in seasonality among populations from different latitudes (Sgolastra, 2007). A variable number of females can nest away from the natal nesting site and emigrate to other places. Pre-nesting dispersal depends on available floral and nesting resources (Bosch et al., 2008). Females of *O. cornuta* have been found nesting 2 km away from their release site (Bosch and Vicens, 2006). This evidence suggests that *Osmia* populations should be able to migrate at least a few km per year and that progeny are likely to disperse to and mate in environments within a range of 10 to 100 kilometers of those experienced by their mothers.

Table: 2.1: Overview table on ecological entities

Ecological Entity	Solitary bees	Social bees
Individual	Single bee	Single bee
Colony	-	Different categories of bees that live in a single hive
Population	Group of males and females (of the same species) nesting in the same place spatially defined by the maximum foraging range. The nests with the brood are in the centre of the foraging area.	Group of colonies which is spatially defined by the mating area
Metapopulation	Multiple bee populations of the same species living in fragmentated habitats connected through immigration and emigration	Multiple bee populations of the same species living in fragmentated habitats connected through immigration and emigration

Colonies are suggested as ecological entities for defining protection goals of honey bees and social non-*Apis* bees for the ecosystem services related to pollination, hive products and biodiversity. For the ecosystem service pollination is suggested to define in addition foragers as the relevant ecological entity. Populations are suggested as ecological entities for solitary non-*Apis* bees.

Meta-populations would be appropriate entities for the specific protection goal based on cultural services. But effects on meta-populations are difficult to assess and therefore it is suggested to use colonies (honey bees, social non-*Apis*) and populations (solitary bees) as a surrogate.

2.2.2 Attributes to protect

The pesticide legislation lists acute and chronic effects on the survival and development of the colonies and effects on larvae and honey bee behaviour as attributes to protect. Although not specifically mentioned in the legislation, it is also proposed to include abundance/biomass and reproduction because they are important parameters for the development and long-term survival of colonies.

2.2.3 Magnitude of effects:

The physiological mortality of a healthy beehive is not easy to ascertain. It depends on numerous variables such as the season, the strength of the colony and the surrounding environment.

Taking into consideration the number of eggs laid by the queen during the season, the number of brood cells occupied, and the number of adult bees, it may be hypothesized that about 1000 – 2000 bees die naturally on any given day during the period of densest population, i.e. from May to July; smaller values are recorded both before and after these months (Chauvin, 1968; Capelo et al., 1983). The calculations made on the basis of the bees collected in cages will be an underestimate because the efficacy of this gathering method varies according to the environment the hives are situated in and the season (Porrini et al., 2002). The daily mortality rate for all bees in a colony was estimated as about 1% based on the following publications; i) Sakagami and Fukuda (1968), used by DeGrandi-Hoffman et al., (1989), Schmickl and Crailsheim (2007), and ii) Gary (1960) cited by Moritz and Southwick (1992). A mortality rate of 1% corresponds to about 400 – 500 honey bees for summer colonies containing 40 000 to 50 000 bees.

It is difficult to count the dead honey bees in the hive, because few honey bees die inside, and the corpse of those which die in the hive are rapidly removed by specialized bees ("undertakers") and thrown up to several hundreds meters away from the hive. The percentage of bees dying in the field while foraging and the percentage of bees dying in the hive is estimated as approximately 90 % and 10 %, respectively (Gary, 1960, 1976).

Disease or pesticide intoxications can result in mortalities largely above the natural background mortality rates suggested above. Nevertheless, when considering foragers' mortalities, in the short-term it could be underscored because if foragers die in the field or on the way back to the hive they would not be collected in the dead bee traps.

A maximum allowable relative rate of bee mortality could be very useful in field tests if it is not possible to compare the bee mortality with a real control (both treatments exposed at the exactly same conditions). Often, only comparisons between pre and post-treatment are possible. Thus, due to the statistical limitations of field tests (see Chapter 5), a reference level of bee mortality in field conditions could be very useful. The reference level used in the Italian monitoring studies is a realistic epidemiological reference but may be difficult to apply in specific toxicological tests (see Appendix B). In fact, the bee mortality level depends on (other than the test substance): dead bee trap, season, colony size, honey bee race, environmental conditions.

It was agreed that negligible effects should be below the natural background mortality (no increase of natural background mortality). Increased mortality has different consequences for the survival of a bee colony depending on the time of the year. In summer a higher mortality rate will not have such severe consequences for the colony as it would in autumn or early spring. Therefore it is suggested to always compare the observed effects with untreated control colonies.

In the article of Khoury et al. (2011) it was demonstrated with modelling of bee colonies that a daily forager mortality rate of 35.5% is the threshold for a stable colony. The model predictions in Khoury et al. (2011) were in line with field data of Ruepell et al. (2009). Higher mortality rates would lead to extinction of the colony. Forager mortality of 30% is the threshold when in-hive bees start shifting to forage earlier to replace dead foragers (Thompson et al., 2007).

A large effect is defined as the daily forager mortality of 35% (where colonies become unstable – see Khoury et al., 2011). Negligible effects should not exceed the natural background mortality of 3.5% forager mortality (3% estimated from Porrini et al., 2002 – see Appendix B.), 3.5% used in Schmickl and Crailsheim, 2007).

It was decided not to use absolute numbers or percentages of mortality to define the magnitude of effects because of the variability of mortality rates depending on the season, health status, climatic conditions etc. Instead, it is suggested to use factors of increase in mortality compared to controls.

There is a factor of 10 between background mortality of 3.5% and the daily forager mortality of 35% when colonies become unstable. Small and medium effects should be in between. The increase in mortality could be compared to control mortality in tests.

Table 2.2: Examples of definitions of magnitude of effects

Magnitude of effect	**Percentage of daily mortality of foragers**	**Factor of increase of mortality (this could be compared to controls in a semi-field or field study**
Negligible effect	3-3.5 % (background mortality is variable and depends also on the season)	1 No increase including up to a certain increase in daily mortality compared to controls which still needs to be defined. 50% increase in mortality (depending on absolute mortality numbers) is usually detectable in a field study based on expert judgement.
Small effect	7 %	2 100% increase in effects compared to control
Medium effect	17.5 %	5
Large effect	30-35%	10

It is recognized that the protection goals given above are likely to be more stringent than can be feasibly defended by conventional field experimentation. For example, one conventional method for measuring mortality in honey bees is to count the number of dead bees found in a special trap placed outside the hive. In hives that are not exposed to toxins, the background level of weekly mortality is noisy. Specifically, it has a coefficient of variation CV = 0.6, which means that the expected difference of the mortality count of any particular hive from the average among hives (i.e. the standard deviation of mortality counts) is 60% of the magnitude of the mean itself. Given this CV, we can calculate the number of hives needed to detect an imposed treatment effect with the conventional level of confidence (i.e. statistical power = 0.8). It requires prohibitively large experiments involving thousands of hives to reliably test whether a protection goal of negligible effect (3.5% effect) has been breached (Fig 1).

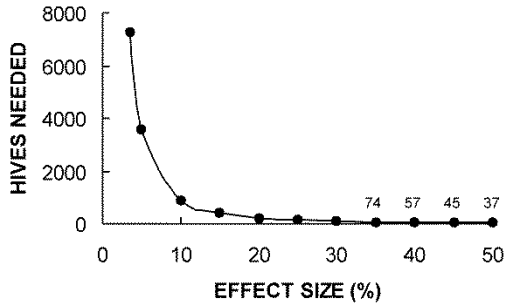

Fig. 2.1: The number of honey bee hives needed to detect specified increases in mortality rate (percentage effect size) using dead bee traps. The CV was calculated from weekly mortalities in bee traps of 24 hives studied in July and August 2008 and 2010 (Helen Thompson, Defra, pers. comm.). Variation among hives was relativized to the mean among hives at that time. Power analysis to calculate the required sample sizes was conducted with **R** statistical software using the following command: `power.t.test(delta = **, sd = 0.6, sig.level = 0.05, power = 0.8, type = "two.sample", alternative = "one.sided")` where '**' denotes the magnitude of the effect (e.g. 0.035, 0.15, 0.35, etc.)

A field-scale experiment using conventional bee traps would require 74 hives to detect an effect of 35% with a significance level of 0.05. In the specific protection goals which are proposed it would be necessary to detect small deviations from the natural background mortality.

Intrinsic variability among hives is a major hindrance to field testing and we therefore recommend that attention be given to developing field-relevant laboratory testing paradigms and to improve the test design of field studies in such a way that small increases in mortality can be detected.

For all other effects (e.g. on behaviour) it is proposed to use the same percentages as for mortality because effects on behaviour could lead to similar consequences for the colony as mortality e.g. if the orientation behaviour of foragers is affected to such an extent that they are not able to return to the hive then the effect on the colony would be the same as if these foragers died.

Data on mortality rates of bumble bees and solitary bees are scarce and it was not possible to give clear definitions for the magnitude of effects based on background mortality and thresholds of effects on populations of solitary bees. The factors were analysed that could increase or decrease the risk in bumble bees and solitary bees compared to honey bees if the same effect percentages are used in the risk assessment as for honey bees.

In order to protect solitary bees it is important to avoid pesticide impacts close to the nest sites including their foraging ranges during their nesting period. For bumble bees the most sensitive period is during the establishment of nests by queens in the spring (Thompson and Hunt, 1999). Chemicals that are highly toxic to bees reduce population size by causing a high mortality rate. However, application of insecticides at sub-lethal doses may also adversely affect nesting behaviour, brood and cell production rate. In solitary bees, the cell production rate (the number of provisioned cells per day) is highly correlated with the fecundity of the nesting females (Bosch and Vicens, 2006) and can be used to estimate the population dynamic. Ladurner et al. (2008) reported that following pesticide applications, females interrupt their nesting activities and spend unusually long periods inside their nests, despite favourable foraging conditions. In this case, cell production rate diminishes dramatically as well as pollination service. By the end of the flowering period, cell production in three sprayed orchards was 108.3 compared with 10,012 in a non-sprayed orchard blooming at the same time in the same area (Ladurner et al., 2008). In this case the number of cells produced in the treated orchard was a factor of 100 lower than in the non treated orchard. Such an effect would be considered as a very large effect. Available data were insufficient to quantify the intermediate magnitude of effects.

The following table gives an overview on factors that could increase or decrease the risk in solitary bees compared to honey bees (see also table 1 in Brittain and Potts, 2011).

Table 2.3: Factors that could increase or decrease the risk in solitary bees compared to honey bees.

FACTORS	EXPECTED PESTICIDE IMPACT	SOLITARY BEES vs HONEY BEES
Body size	Small bees are more sensitive than large ones due to the high ratio surface/volume	+/-: the body size of solitary bees ranges from 1.5 to 46 mm in length. The body size of honey bees is about intermediate.
Nesting period	Bees can dramatically reduce their population if the insecticide applications coincide with the nesting period. Vice versa is low.	+: many solitary bees have a short nesting period (1-2 months) compared with the queen oviposition activity in honey bees (8-9 months depending on climatic conditions).
Foraging range	Risk is higher if the insecticide application point is close to the foraging range. Vice versa is low.	+: solitary bees show shorter foraging range than honey bees. Honey bees can dilute the effect by foraging in other habitats.
Floral specialization	Polylectic species can collect pollen and nectar in a large variety of plants. In this case the pesticide impact may be diluted by bees foraging in other plants not contaminated with pesticide. Risk higher in oligolectic bees if they forage in or near plants where pesticide is applied.	+: honey bees are polylectic and can dilute the effect by foraging in other plants. Many solitary bees are oligoletectic.
Nesting location and nest construction	Risk is higher if the nest site or nesting material is in or near the area of pesticide application	+: Solitary bees can nest in treated fields and can use several nesting materials (mud, leaves, resin) contaminated with pesticide. For honey bees it is possible move the hives from in-field to off-field.
Population size	The risk is higher in smaller population (e.g. 50% of mortality has smaller impact in a large population of 10000 individuals than in a population of 200 individuals).	+: Often the population size in solitary bees is very small.
Voltinism and flight season	If the flight season of the species coincides with the application time of the insecticide then the impact will be higher, in particular in the univoltine species since the bivoltine and multivoltine species can spread the risk across the year	-: In solitary bees there are univoltine and multivoltine species. In this case the risk is higher only for the univoltine species when the application time coincides with the flight season. Instead in honey bees the risk is higher given their long flight season.
Sociality	In social bees the risk can be mitigated by high number of workers if the application time doesn't affect the queen's activity. In solitary bees the risk is always high if the application time coincides with the nesting female period.	+/-: In solitary bees the risk is high if the flight season of the species coincides with the application time. In honey bees and other social bees the risk is high if the colony founder is active (in honey bee: queen during mating flight or swarming) during application time.

+: risk potentially higher in solitary bees; -: risk potentially lower in solitary bees and higher in honey bees.

The overview table on the different factors influencing the vulnerability to pesticides suggests that solitary bees could be more vulnerable to impacts from pesticides compared to honey bees. There is uncertainty regarding the extent to which each of the factors would influence the vulnerability to pesticides. It may be that the effect percentages proposed for honey bees are not protective enough for all species of solitary bees, particularly if a product is applied during the nesting period of solitary bees. As a pragmatic solution, effect percentages as for honey bees could be used and an additional safety factor should be included in the risk assessment in cases where a product is applied during the nesting period of solitary bees.

The following table gives an overview of factors that could increase (+) or decrease (-) the risk in bumble bees compared to honey bees (see also Thompson and Hunt, 1999).

Table 2.4: Factors that could increase or decrease the risk in bumble bees compared to honey bees.

FACTORS	EXPECTED PESTICIDE IMPACT	BUMBLE BEES vs HONEY BEES
Body size	Larger bees are less sensitive than smaller ones due to the lower ratio surface/volume	-: the body size in bumble bees is larger than that of honey bees although highly variable within a species and between species.
Nesting period	Bees can dramatically reduce their population if the insecticide applications coincide with the nesting period.	+: many bumble bees show short nesting periods (1-2 months) compared with the queen oviposition activity in honey bees (8-9 months depending on climatic conditions).
Foraging range	Risk is higher if the insecticide application point is close to the foraging range. Vice versa is low.	+: bumble bees have a shorter foraging range than honey bees. Honey bees can dilute the effect by foraging in other habitats.
Floral specialization	Polylectic species (honey bees) can collect pollen and nectar in a large variety of plants. In this case the pesticide impact may be diluted by bees foraging in other plants not contaminated with pesticide. Risk higher in oligolectic bees if they forage in or near plants where pesticide is applied.	+: Many bumble bee species are oligoletectic, e.g. short tongued vs long tongued.
Nesting location and nest construction	Risk is higher if the nest site is in or near the area of pesticide application.	+: Bumble bees can nest on the edges of treated field (unlikely to be within field due to field husbandry) and may be subject to migration of the pesticide through the soil to nests in soil nesting species. For honey bees it is possible move the hives from in-field to off-field.
Colony size	The risk is higher in smaller colonies (e.g. 50% of mortality has smaller impact in a large colony of 1000 individuals than in a colony of 100 individuals).	+: Often the colony size in bumble bees is small and varies from less than 100 to 400 individuals depending on species.
Voltinism and flight season	Only queen bumble bees overwinter. The establishment of nests by queen bumble bees occurs over a relatively short period and pesticide impacts can cause loss of queens. The timing of queen establishment of nests depends on species.	+: if pesticide impacts occur on the foraging queen during colony establishment.

Sociality	In social bees the risk can be mitigated by a high number of workers if the application time doesn't affect the queen's activity.	+: In bumble bees the risk is high if the establishment of the colony by the queen of the species coincides with the application time. This occurs over a longer period of time than the mating period of a queen honey bee.

+: risk potentially higher in solitary bumble bees; -: risk potentially lower in bumble bees and higher in honey bees.

Most factors listed above suggest a higher vulnerability of bumble bees compared to honey bees, in particular if the pesticide application coincides with colony establishment and exposure of the queen. The larger body size of bumble bees makes them less susceptible to pesticides compared to honey bees. It is unclear if this would fully compensate the higher vulnerability with regard to other factors such as smaller colony size, potentially higher exposure and shorter nesting periods. The risk to bumble bees may be underestimated if the same magnitudes of effects as for honey bees are applied in the definition of protection goals. As for solitary bees, an additional assessment factor may be applied in the risk assessment to account for this uncertainty if the same level of protection as for honey bees is desired.

2.2.4 Temporal scale:

Days – how many days still needs to be defined

Weeks – up to 3 weeks (one brood cycle for honey bees)

Months – up to 3 months (4 brood cycles for honey bees)

Seasons – up to 4 seasons (12 months)

Years – more than 1 year

The temporal scale of days is proposed to be used in the specific protection goal in combination with small effects. Small effects are defined for example as a daily forager mortality rate of 7%. It was not possible on the basis of the available information to determine the timespan for which an increase in mortality to 7% would lead to severe effects on the hive. Based on expert judgement of the working group, it was considered that such an increase in mortality could be tolerated for a few days without putting the survival of a hive at risk. Further research (modelling) is proposed to clarify this question and to revise the proposal for the temporal scale of effects.

2.2.5 Proposal for specific protection goals

The final decision on protection goals needs to be taken by risk managers. There is a trade-off between plant protection and the protection of bees. Therefore it may be necessary to accept a certain level of effects at least in the in-field area where products are applied. The protection goals proposed below would provide a very high level of protection to bees. The overall level of protection also includes the exposure assessment goals. Decisions need to be taken on how conservative the exposure estimate should be and what percentage of exposure situations should be covered in the risk assessment (e.g. should the exposure estimate be an average or should it cover 90% or more of all exposure situations). For exposure assessment goals see section 2.3.

2.2.5.1 Honey bees:

In-field, pollination service of crop plants,

Ecological entity: Foragers of a colony / colony

Attributes: Behaviour of foragers, survival, abundance

Magnitude: Negligible effects up to small effects / negligible effects on colonies

Temporal scale: days for small effects

Medium effects or even large effects could not be tolerated even for a short period of time (days) because medium to large effects would have impacts on the pollination service (although the colony may survive a medium effect that only lasts up to few days)

Off-field, pollination of non-crop plants

Ecological entity: Foragers of a colony / colony

Attributes: Behaviour of foragers, survival, abundance

Magnitude: negligible effects

Temporal scale: not relevant

In-field and off-field, Food provision service - hive products

Ecological entity: colony

Attributes: behaviour, survival/growth, abundance/biomass, reproduction

Magnitude: negligible effects,

Temporal scale: not relevant

It is proposed to focus the risk assessment on behaviour, survival/growth, aboundance/biomass and reproduction of the colony. All life stages need to be considered in the risk assessment (including in hive bees, overwintering bees, larvae, queens, drones and swarms). It does not necessarily mean that all life stages would need to be tested but it needs to be ascertained whether the risk assessment covers all of them.

Landscape scale, ecosystem services genetic resources, education, aesthetic values

Ecological entity: colony

Attributes: behaviour, survival/growth, abundance/biomass, reproduction

Magnitude: negligible effects,

Temporal scale: not relevant

The protection goal is based on the colony with a focus on behaviour, survival/growth, abundance/biomass and reproduction which is similar to the protection goal for hive products except that it is defined for the landscape scale. This would have consequences for the exposure estimates which are different for landscape scales and would be less conservative compared to the edge of field estimates. It is assumed that the protection goal for genetic resources, education, aesthetic values is covered by the protection goal for hive products.

2.2.5.2 Non-*Apis* bees:

In-field, pollination service of crop plants,

Ecological entity: Colony (bumble bees), Population (solitary bees)

Attributes: Behaviour of foragers, survival, abundance, reproduction

Magnitude: Negligible effects up to small effects

Temporal scale: days for small effects

Off-field, pollination service of non-crop plants,

Ecological entity: Colony (bumble bees), Population (solitary bees)

Attributes: Behaviour of foragers, survival, abundance, reproduction

Magnitude negligible effects

Temporal scale: not relevant

Landscape scale, ecosystem services genetic resources, education, aesthetic values

Ecological entity: Metapopulation, Colony (bumble bees), Population

Attributes: behaviour, survival/growth, abundance/biomass, reproduction

Magnitude: negligible effects on metapopulation, small effects on populations or colonies (bumble bees)

Temporal scale: less than 3 weeks for small effects,

Since effects on metapopulations are difficult to assess, it is proposed to focus on colonies and populations in the risk assessment. The protection goals need to be set in a way that the effects on colonies/populations do not affect metapopulations.

The protection goals for pollination service are more conservative compared to the specific protection goal for genetic resources, education and aesthetic values.

2.3 Definition of the exposure assessment goals related to the Specific Protection Goals

2.3.1 Introduction

EFSA (2010) states that an appropriate definition of the Ecologically Relevant type of Concentration (ERC) is needed to link exposure and effects. Bees can be exposed in different ways (e.g. consumption of pollen and nectar, consumption of guttation water, inhalation via the vapour phase). Therefore the best linking between exposure and effects is likely to be achieved by considering the body burden of individual bees (which may of course also be influenced by the behaviour of the bees) using TK/TD modelling. However, use of the body burden as the type of ERC is at this moment not yet possible in view of the available knowledge. So therefore as a surrogate for the body burden, a number of different ERCs will be considered, as will be described later (concentration in pollen entering the hive, concentration in nectar in hive, concentration in guttation water of crop, etc).

Furthermore EFSA (2010) states that the following spatio-temporal aspects of the exposure assessment goal have to be defined: (i) the type of spatial unit, (ii) the spatial statistical population of these units, (iii) the multi-year temporal statistical population of concentrations for each unit, (iv) the percentile of the spatio-temporal statistical population of concentrations. As described by EFSA (2010), these are essential specifications of the protection-goal dimensions because the risk is only assessed for the spatio-temporal variability of the systems that are included (e.g. if a 90^{th} percentile of the spatio-temporal population of concentrations is considered in the exposure assessment, then for the remaining 10% of the systems the exposure concentrations are higher than considered in the exposure assessment and so for these 10% remaining exposure situations effects may occur (and this is considered acceptable by the risk managers). However, the realisation of effects for individual substances depends on the toxicity to bees in combination with the actual application rates and the margin of safety that is indicated for the individual substance.

This exposure assessment goal refers to a certain plant protection product and the combination(s) of application method, application rate, application time window and crop or crop groups as specified by the notifier in his authorisation submission (e.g. spray applications of insecticide X to be applied in sugar beets once per growing season at a rate of 1 kg/ha between crop development stage Y and Z).

The different specific protection goals (SPGs) described above need partly different types of spatial units and different spatial and temporal statistical populations (e.g. in or off field). Therefore these aspects of the exposure assessment goals are described below for each of the SPGs.

2.3.2 Type of spatial unit and spatial statistical population of units

2.3.2.1 Honey bees

The aim of the exposure assessment is a certain percentile of a concentration in the space-time continuum. This requires that the elements of the statistical population of this concentration are defined. The first step is to define the type of spatial unit to which the concentration refers. As described in the previous section the best link between exposure and effects is likely to be achieved in the future by using the body burden of bees as the type of ERC. So the type of spatial unit has then to be either an individual bee or a group of bees.

We use acronyms for the different SPGs as explained in Table 2.3. For AP-I-PC and AP-O-PNC the type of spatial unit is an individual forager bee. For AP-IO-HP and AP-IO-GEA the type of spatial unit is a colony.

The first aspect of the spatial statistical population is the total area to be considered. For example, this could be the whole EU, one of the regulatory zones North-Centre-South or a Member State. In view of

the terms of reference, we propose to consider each of the regulatory zones North-Centre-South as the total area for all SPGs.

The second aspect of the spatial statistical population is the location of the spatial units in the landscape in relation to the application of the substance. For AP-I-PC only the forager bees within treated fields are considered. For AP-O-PNC the Panel proposes to consider forager bees at the edge of treated fields. The alternative would be to consider forager bees at the landscape level for AP-O-PNC but this would introduce a large fraction of unexposed forager bees in the exposure assessment and thus lower the level of protection. For AP-IO-HP and AP-IO-GEA examples of the locations of hives in the landscape are (i) in agricultural fields treated with this plant protection product, (ii) at the edge of agricultural fields treated with this plant protection product, (iii) anywhere in the landscape, etc. The Panel considers hives at the edge of treated fields the most relevant exposure assessment goal for AP-IO-HP because hives are usually not places within treated fields. For AP-IO-GEA the Panel also proposes to consider hives at the edge of treated fields because this will avoid 'dilution' of the spatial population with a large fraction of unexposed hives. Table 2.3 gives an overview of the proposed types of units and their locations in relation to the application of the substance.

Table 2.5: Overview of the proposed types of spatial units and their locations for the SPGs of the honey bees (*Apis mellifera*).

Specific Protection Goal	Acronym	Type of spatial unit	Location of unit
In-field, pollination service of crop plants	AP-I-PC	Individual forager bee	In treated fields
Off-field, pollination of non-crop plants	AP-O-PNC	Individual forager bee	At edge of treated fields
In-field and off-field, food provision service - hive products	AP-IO-HP	Colony in hive	At edge of treated fields
In-field/off-field, landscape scale, ecosystem services genetic resources, education, aesthetic values	AP-IO-GEA	Colony in hive	At edge of treated fields

2.3.2.2 Non-*Apis* bees

We use acronyms for the SPGs as specified in Table 2.4. The type of spatial unit proposed for NAP-I-PC and NAP-O-PNC are individual bumble bees and individual solitary bees. For NAP-IO-GEA the type of spatial unit is a colony for the bumble bees and a population for the solitary bees.

The Panel proposes to consider each of the regulatory zones North-Centre-South as the total area for the spatial statistical population for all SPGs. Another aspect of the spatial statistical population is the the location of the spatial units in the landscape in relation to the application of the substance. For NAP-I-PC, the Panel proposes to consider all bumble bees and solitary bees within treated fields because this is most relevant for the pollination of crop plants. For NAP-O-PNC and NAP-IO-GEA the Panel proposes to consider all types of spatial units at the edge of treated fields (in analogy with AP-O-PNC and AP-IO-GEA as shown in Table 2.3). Table 2.4 gives an overview of proposed types of units and their locations.

Table 2.6: Overview of the proposed types of spatial units and their locations for the SPGs of the non-*Apis* bees.

Specific Protection Goal	Acronym	Type of spatial unit	Location of unit
In-field, pollination service of crop plants	NAP-I-PC	Individual bumble bees and individual solitary bees	In treated fields
Off-field, pollination of non-crop	NAP-O-PNC	Individual bumble bees	At edge of treated fields

plants		and individual solitary bees	
In-field/off-field, landscape scale, ecosystem services genetic resources, education, aesthetic values	NAP-IO-GEA	Colony of bumble bees and population of solitary bees	At edge of treated fields

2.3.3 Multi-year temporal statistical population of concentrations

The previous section described a number of different types of spatial units at different locations (see Tables 2.3 and 2.4). The definition of the multi-year temporal statistical population of concentrations is not the same for all these different combinations of types of units and their locations. Let us first consider the potential complexity of an application sequence of a certain plant protection product. Consider the following example of a complicated but not unrealistic application sequence of a certain plant protection product:

- year 1: 1 kg/ha in maize and 0.5 kg/ha in carrots
- year 2: 0.7 kg/ha in peas
- year 3: no applications
- year 4: no applications
- year 5: 1 kg/ha in maize and 0.5 kg/ha in carrots
- year 6: 0.7 kg/ha in peas
- … etc

We use such a complicated application sequence to ascertain that the specification of the multi-year population of concentrations takes into account all possible combinations of uses that may occur in regulatory practice. For individual bees in treated fields (AP-I-PC and NAP-I-PC in Tables 2.3 and 2.4), it is most appropriate to base the population of concentrations only on the application year in the sequence that is likely to result in the highest exposure (which will depend on the combination of the dosage and the crop). If we assume that the dosage of 1 kg/ha in maize gives the highest exposure, then the multi-year temporal population is based only on years 1 and 5 (and thus the other years that generate lower or zero values are ignored to avoid 'diluting' the population with lower or zero values).

For AP-IO-HP and AP-IO-GEA it does not make sense to construct a multi-year statistical population of concentrations in hives for such an application sequence because a hive is likely to be linked to only one of these crops (which may not be the case for e.g. a colony of social non-*Apis* bees which lives in the same field for some years or for the aquatic ecosystem in a ditch at the edge of a field with this application sequence). Thus for AP-IO-HP and AP-IO-GEA the multi-year statistical population of concentrations is based on the hives that are at the edge of fields grown with the single crop for which the risk assessment is carried out. In the case of the above application sequence, this would mean that only the dosage-crop combination that generates the highest exposure is considered.

For NAP-O-PNC and NAP-IO-GEA (Table 2.4) it is most appropriate to base the population of concentrations only on the application year in the sequence that is likely to result in the highest exposure because colonies of bumble bees and populations of solitary bees do not follow the crops as do the hives of honey bees.

2.3.4 Which percentile of the spatio-temporal statistical population of concentrations?

The definition of any percentile has to include both space and time. Let us consider as an example AP-IO-HP and let us consider all the hives at the edge of one treated field as a single entity (called 'the hive'). Let us assume that we have a population of 100 hives at the edge of 100 fields grown with the crop at stake and a time series of 20 years of concentrations in each of these hives (placed at the edge of treated fields). Note that a field is not fixed to one location in this context. For example, for an exposure assessment of an insecticide application in maize in the Centre zone, the population consists

each year of all hives at the edge of the maize fields in the Centre zone where this insecticide is applied.

Let us further consider for example as ERC the annual peak concentration in pollen reaching the hive. This annual peak concentration will vary in the same year between these 100 fields even if the climatic conditions are exactly equal in these fields because, for example, the landscape surrounding the fields will differ. This peak concentration will also vary from application year to application year at the edge of a single field grown with this crop, for example, because the weather may influence the concentration in the pollen and the activities of the bees.

Following the above example, we have a statistical population of 100 x 20 = 2000 peak values. If we knew these 2000 values, a contour diagram could be constructed as shown in Figure 1. The procedure to make such a contour diagram is as follows: first for each field a cumulative frequency distribution is made of the 20 concentrations. Then the fields are ranked based on the concentration at the 50th percentile in time. Then the 2000 concentrations are transformed into percentiles and plotted as a contour diagram. So we find at the left the fields with the lowest concentrations and at the right the fields with the highest concentrations. At the bottom we find the years with the lowest concentrations and at the top the years with the highest concentrations. Usually a certain percentile is a continuous line in such a diagram. In Figure 1 the pink line shows all space-time combinations that correspond to the 90th percentile concentration as an example. In this approach no distinction is made between space and time with respect to the consequences of the risk assessment: e.g. the following two cases are considered equally undesirable:

1. the concentration exceeds the acceptable level in hives at the edge of 20 fields in one application year

2. the concentration exceeds the acceptable level in 20 application years in hives at the edge of one field.

It is of course possible to specify further restrictions to the spatio-temporal percentile based on the specific protection goal: e.g. it may be undesirable that hives suffer from unacceptable effects year after year at the same spot or in a certain region.

The procedure as described above can in principle be applied to any ERC for any of the specific protection goals as described above.

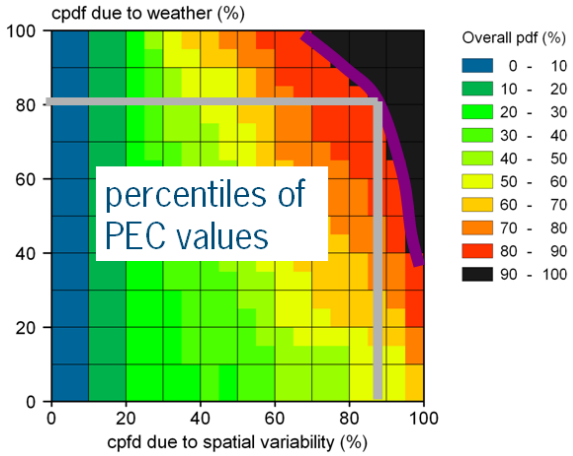

Figure 2.2: Hypothetical example of contour diagram of percentiles of exposure concentrations as a function of the spatial and temporal percentiles. The pink line shows all combinations that give an overall 90th percentile and the grey lines show a possible combination of a spatial and a temporal percentile giving an overall 90th percentile.

The value of the percentile is of course a risk management choice. In the past, the SCFCAH agreed to 90th percentiles for the assessment of the risk of leaching to groundwater and exposure of aquatic organisms which the SCFCAH considers realistic worst cases. However, this is a subjective link because a 99th percentile is of course also a realistic worst case. The value of the percentile is linked to the required overall level of protection for bees; this has so far not been defined by the SCFCAH.

The highest level of protection would of course be achieved by prescribing use of the 100th percentile of the spatio-temporal statistical population of concentrations. Admittedly, then most of the specifications in the previous sections are irrelevant (the location of the hive in the landscape in relation to the application of the substance, the definition of the multi-year temporal population of concentrations , spatio-temporal combinations) because the 100th percentile is the highest single value that will ever occur resulting from the specified use of the plant protection product in the specified regulatory zone. However, use of a 100th percentile for the exposure is likely to lead to the conclusion that the SPGs are not met for many of the currently registered insecticides because the 100th percentile exposure is of course an extremely unlikely unfavourable case (e.g. a case where generation of guttation water containing high concentrations coincides with a high water demand of the hive without any other water source in the surroundings).

Let us consider possible consequences of choosing an exposure percentile of less than the 100th. In the case of the AP-IO-HP specific protection goal (colonies in hives), selection of a 90th percentile would have the consequence that the SCFCAH considers it as acceptable that 10% of the hives suffer from non-negligible effects each year. This could mean that there are negative effects on for example honey production in 10% of the hives each year for a certain period of time or decrease in pollination service which could lead to lower yields in insect pollinated crops.

For exposure of humans to pesticide residues via the diet, the SCFCAH uses a 97.5th percentile as suggested in the FAO manual (FAO, 2009). One could argue that it is difficult to defend to use a more strict exposure assessment goal for bees than for humans, thus using a lower percentile than the 97.5th. However, the Panel considers this not an appropriate way of reasoning because the overall level of protection is given by the combination of the SPG and the exposure assessment goal. Thus it does not make sense to discuss exposure percentiles in isolation from the effect assessments.

It is an option to choose a higher percentile for the honey bees than for the non-*Apis* bees in view of the high societal value of healthy bee hives. For example use a 90th percentile for the non-*Apis* bees in analogy to the 90th percentile used for the aquatic organisms and a 95th percentile for the honey bees.

In view of these considerations, the Panel does not propose a certain value of the percentile but instead will use the terminology of 'the xth percentile' assuming a value somewhere between the 90th and 95th percentile. The Panel will seek the advice of the SCFCAH on the value of the percentile to be used in combination with the proposed SPGs.

2.3.5 Consequences for the exposure assessment

As described before, the risk assessment applies to a certain plant protection product and the combination(s) of application method, application rate, application time window and crop or crop groups as specified by the notifier in his authorisation submission. The exposure assessment goal is thus the 95th percentile of the spatio-temporal concentration distribution for this combination of application method, application rate and application time window in one of the three regulatory zones in the EU.

The risk assessment for aquatic and soil organisms is based on parallel tiered approaches for the effect and exposure assessment enabling the risk assessor to choose to go either to a higher tier in the effect assessment or to a higher tier in the exposure assessment (EFSA, 2010, 2012). The risk assessment for bees consists of tiered approaches in which the exposure and effect are integrated. In the higher-tier studies usually only the effect of a single treatment level is compared to an untreated control. So this

has the consequence that the exposure in any higher tier effect study for bees has to be higher than that corresponding to this 95th percentile.

Let us consider for example the risk assessment for the SPG AP-IO-HP (honey bee colonies in hives at edge of treated fields) and for a spray application of an insecticide at a rate of 2 kg/ha in maize in the Centre zone at a certain crop development stage. Let us assume that the higher-tier field study was carried out in the UK. The exposure assessment issue is then whether the exposure in this UK field study was indeed higher than will occur for this 95th percentile of the spatio-temporal distribution of concentrations in hives at the edge of treated maize fields in the Centre zone resulting from this application.

So for such an assessment there has to be agreement on the relevant types of ERCs and on the main factors that influence these types of ERCs. Preferably a decision flow chart should be designed for checking whether exposure in the field study is indeed higher than that corresponding to this 95th percentile (assuming good agricultural practice). These main factors may include for example:

- the crop and its development stage in the field study and the crop and its development stage in the agricultural reality,
- the dosage in the field study and the dosage in reality,
- measures taken to ensure contact between bees and crop in the field study (spraying of sugar solution, crop and hive in the same tunnel),
- the location of and weather conditions at the field study to be compared with the range of conditions in the zone for which the risk assessment is carried out
- the generation of guttation water by the crop in the field study compared to the generation of guttation water by the crop in reality.

3 CHAPTER 3: EXPOSURE

3.1 Summary

In this chapter, bees' exposures to contaminated products through ingestion, contact and inhalation were determined by compiling available quantitative data on food intake (e.g. nectar, honey, honey dew, pollen, water), nesting material (e.g. wax, propolis) and residues found in bee products (e.g. nectar, honey, pollen, wax) and in various other materials in contact with bees (e.g. water on plants, in the field and in the air, dust particles during sowing operations, residues in soil, etc.).Based on this information, a qualitative assessment was made by expert judgement, to determine the most critical categories of bees to be further considered in the risk assessment scheme.

Highest concentrations following spray applications compared to seed treatments were found in nectar and pollen. Exposure to residues in guttation droplets and oral contact exposures following dust drift were investigated. These data showed a wide variability due to the number of parameters known to influence guttation production (environmental conditions, crop type, growth stage, etc.). However, a potential high exposure was highlighted for bees exposed to residues from guttation droplets in some crops (e.g. in maize).

The analysis of the factors influencing dust exposure (i.e. dust formation and emission data and residue data in/on collectors, e.g. petri dishes, neighbouring crops and bees exposed to dusts) demonstrated that dust drift is a potential relevant exposure route during sowing of treated seeds. Several factors are influencing the level of exposure: the amount of dust formation during application, the pesticide concentration in the dust and the effect of the abrasion of the seeding machine. Also direct contact with droplets in the spray cloud and the spray drift droplets could result in considerable exposure rates. The use of highly volatile pesticides (e.g. fumigants) could also result in direct exposure to the gaseous emissions of these chemicals.

The risk posed by pesticides to honey bees showed that foraging bees, winter bees and larvae were the most exposed category of bees via oral exposure. However, nurse bees were also identified as potentially highly exposed when considering simultaneous oral exposures via pollen and nectar. The unknown respective amounts of water consumed by foragers (whether coming from puddles, surface, leaves and/or axils) and the unknown amount and fate of water inhaled by in-hive bees did not allow us to characterise these risks. Larvae in contact with wax and foragers, drones, queens and swarms intercepting droplets and vapour in/out field were found to be the most exposed categories of bees via contact and inhalation exposures, respectively.

The risk posed by pesticides to bumble bees indicated that workers, queens and larvae were the most exposed categories of bees via oral exposure. Because most species nest in the soil, exposures by contact and inhalation (from soil fumigant) were considered highest for all categories of bees. Exposures by contact and inhalation of droplets and vapour in/out field were considered maximal for workers, drones and queens.

The risk posed by pesticides to solitary bees by oral exposures was considered highest for adult females and larvae. Adult females and larvae showed maximum exposure by contact with soil, foliar residues or other nesting material. Exposure by contact and inhalation were considered maximal for adult females and males with interception of droplets and vapour in/out field. Exposure by inhalation (soil fumigant) was found to be highest in nesting females and larvae for species nesting in soil.

It was concluded that for the ranking of bees, the inclusion of multiple exposures with appropriate weights would need to be done with a modelling or scenario-based approach that was not available in the current assessment. It was therefore recommended that the categories of bees which represent the worst-case exposure scenarios through multiple exposures be further assessed (e.g. honey bee nurses) and that those categories which highlighted potential but unknown exposures through consumption of water and inhalation of vapor in/out field are further analysed with more studies. Finally, as the

database on exposure to residues from different exposure routes for bees is limited, further research is recommended on the testing of the presence and fate of residues (e.g. in bee relevant matrices and in-hive following spray and dust applications) and on the development of reliable exposure models.

3.2 Introduction

The potential routes of exposure to pesticides in bees (honey bees, bumble bees and solitary bees) were identified. In particular, plant (nectar, pollen, guttation water, propolis and plant surface such as leaves, petioles, axils, etc.) and non-plant (droplets of spray and solid particles in air, water and/or soil compartments) routes of exposure were analysed. Exposure to sprayed and non-sprayed (systemic and non-systemic) insecticides and soil fumigants were investigated. For all these modes of applications, exposure through oral, contact and inhalation were assessed as well as exposure through contact with, and inhalation of, dusts from systemic and non-systemic insecticides.

Figure 3.1 summarises the major routes of exposure of foraging bees to pesticides applied to crops which may be returned to the colony including exposure to puddles which was not represented on the figure. The diagram is illustrated by the honey bee but is applicable to bumble bees and solitary bees with modifications, e.g. in solitary bees foragers are replaced by queens and the hive only exists for honey bees.

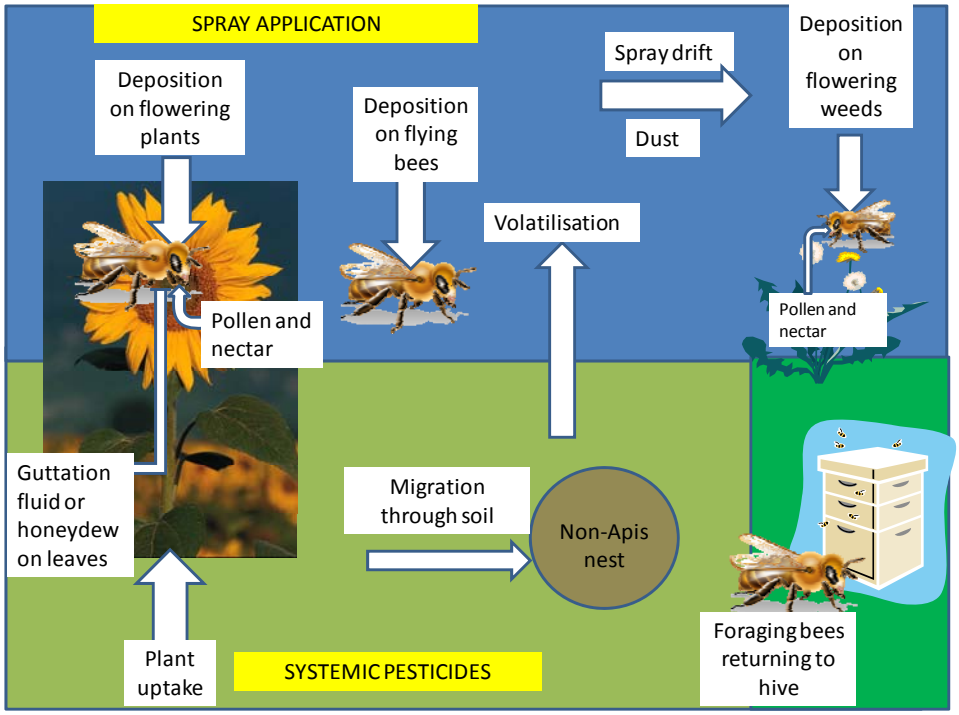

Figure 3.1: Major routes of exposure of foraging bees to pesticides.

Honey bees' exposure was determined for foragers, wax producing bees, nurse and brood-attending bees, winter bees, mating drones and queens, swarms and larvae. Bumble bees' exposure was estimated for workers, drones, queens and larvae and solitary bees' exposure was assessed for adult females, adult males and larvae.

Oral exposure was estimated by determining the amount of food (nectar, honey, honey dew, water and pollen) required by bees to achieve their daily tasks. Contact exposure was determined by estimating the amount of plant and non-plant material in contact with bees (pollen, soil, plant surfaces, water, dusts and droplets for all types of bees; propolis for honey bees and wax for honey bees and bumble bees), while exposure by inhalation was assessed through dust and vapour in the field. Bees' exposure and residue concentrations in plant and non-plant material were determined with the available data

found in literature. For estimation of acute and chronic exposure of bees to residues in nectar, honey, pollen and bee bread following spray applications and systemic seed treatments, residue data from published literature, Draft Assessment Reports (DARs) and bee monitoring were compiled. Based on these estimates, the risk posed by pesticides to bees was finally determined qualitatively by expert judgement with a ranking method (scores from "0" to "4" corresponding to "no exposure" to "highest exposure") and taking into account possible multiple exposures.

3.3 Potential exposures via plant routes

3.3.1 Honey bees' exposure

The various food (e.g. pollen, nectar, water) and bee products (e.g. propolis) ingested or in contact with bees have been estimated from literature and are detailed in Appendix A. These estimates were calculated for the different categories of bees (i.e. nectar, pollen and water foragers, wax producing bees, nurse and brood attending bees, winter bees, drones, queens, swarms, drone, queen and worker larvae). The food intakes calculated by Rortais et al. (2005) served as a basis for further implementation with new categories of bees and new estimates added (see Table 3.1).

Table 3.1: Individual honey bees' food intake (sugar from nectar, honey and honey dew and pollen from flowers and bee bread) and contact with bee products (pollen, propolis, water)

		A	B	C	D	E	F	G
	Products\ Categories of bees	Foragers (a: nectar/b: pollen/c: water)	Wax pro-ducing bees	Nurse/ brood attendi ng bees	Winter bees	Drones	Queens	Larvae (a:workers/ b:drones/c: queens)
1	Sugar in nectar from flowers (oral exposure)	a: 32-128mg/d b-c: NR	NR	NR	32-128mg/d (1Aa, foraging)	NR	NR	NR
2	Sugar in nectar stored in the hive (oral exposure)	a: ≥32-128mg/d b: ≥10-16mg/d c: 72-110mg/d	18mg/d	34-50mg/d	8.8mg/d (thermo) or 34-50mg/d (2C, brood) or 32-128mg/d (1Aa, foraging)*	21-90mg/d	42-81mg/d)	a: 59.4mg/5d b: 98.2mg/6.5d c: unknown
3	Pollen from flowers (contact exposure)	a: unknown b: 150mg/d 300mg/d c: NR	5-10% pollen in wax and propolis	≥ 6.5-12mg/d (4C, brood) and 5-10% pollen in wax and propolis	150mg/d (3Ab, foraging) or 6.5-12mg/d (4C, storing) and 5-10% pollen in wax and propolis	Negligible amounts of pollen in wax and propolis	Negligible amounts of pollen in wax and propolis	Negligible amounts of pollen in wax and propolis
4	Pollen from bee bread (oral exposure)	NR	NR	6.5-12mg/d	6.5-12mg/d	0.36mg/d (only during the first days after emergence)	NR	a: 1.5-2mg/5d b: 2.04-2.72mg/6.5d c: NR
5	Sugar in honey dew from plants (oral exposure)	a: 32-128mg/d b-c: NR	NR	NR	32-128mg/d (1Aa, foraging)	NR	NR	NR
6	Sugar in honey dew in the hive (oral exposure)	a: 32-128mg/d b: 10-16mg/d c: 72-110mg/d	18mg/d	34-50mg/d	8.8mg/d (thermo) or 34-50mg/d (2C, brood) or 32-128mg/d (1Aa, foraging)*	21-90mg/d	42-81mg/d	a: 59.4mg/5d b: 98.2mg/6.5d c: unknown
7	Water (droplets on leaves, axils, puddles in field, surface water) (contact and oral exposure)	a-b: NR c: 1.4-2.7ml/d	20-42L/colony/year and during summer up to 20L/week/colony or 2.9L/d/colony					
8	Propolis	30-300mg/d	Negli-gible amounts of pollen in propolis	Negli-gible amounts of pollen in propolis	Negligible amounts of pollen in propolis	Negligible amounts of pollen in propolis	Negligible amounts of pollen in propolis	NR

(NR): not relevant; (d): day; (*): during winter, bees will achieve thermoregulation; towards the end of winter, if conditions are good, bees will start foraging and feed the new brood.

3.3.2 Bumble bees' exposure

Table 3.2 shows the uptake rates and total volumes of nectar of varying concentrations, taken by different bumble bee species. It also gives the range of mass measured, to give an indication of size. The results of this work showed that the uptake rate of sugar solution by bumble bees generally slows with increasing concentration - as the solution becomes more viscous. Maximum rates of uptake of solution were found to be in the range 40 - 50 % w/w[8] concentration.

Table 3.2: Nectar uptake in different bumble bee species

Bumble bee species/caste	**Nectar uptake rate (µl/s)**	**Total volume nectar taken (µl) per visit**	**range of unfed mass (mg)**
B. hortorum - workers/gynes	0.9 - 3.0		
B. pascuorum - workers		36.0 - 65.1	74 - 165
B. pratorum - workers	0.3 - 1.8		83 - 160
B. pratorum - gynes	0.5 - 3.1		325 - 425
B. terrestris - workers	0.5 - 2.0	41.1- 111.9	109 - 300
B. terrestris - gynes	N	104.0	
A mellifera		50[a]	100[b]

Data modified from Prys-Jones and Corbet (1991), [a] Crane (1990) and [b] NBU data (Thompson and Hunt, 1999)

Table 3.3 shows the intake of pollen and nectar by worker and larval bumble bees. These estimates are based on the work of Tasei and Aupinel (2008), Tasei et al. (2000), Pereboom (2000) and Tasei et al. (1994).

Table 3.3: Intake of pollen and nectar by worker and larval bumble bees.

	Workers mg/individual/day		**Larvae mg/individual/day**	
	Sugar	Pollen	Pollen	sugar
Consumptions	73-149[b, c]	26.6-30.3[a, c]	22-23[a(for male larvae), d]	23.8[d]

(a) Tasei and Aupinel (2008), (b) Tasei et al. (1994), (c) Tasei et al. (2000), (d) Pereboom (2000)

Further details on the estimated food intake of bumble bees or the amount of food material in contact with bumble bees (adults and larvae) are described in Appendix B.

3.3.3 Solitary bees' exposure

The group of solitary bees comprises thousands of species with different life cycles, behavioural, morphological and physiological features. However, of all the bee species described worldwide, the biology is well known only for few species and only for these is it possible to quantify the exposure. The various foods (nectar and pollen) ingested and the nesting materials (e.g. mud, leaves) in contact with bees have been estimated from literature in two species of solitary bees: the European mason bee (*Osmia cornuta*) and the alfalfa leafcutting bee (*Megachile rotundata*) (see Tables 3.4 and 3.5). These species are reared for crop pollination and many data are available in literature on their life cycle and behaviour.

Table 3.4: Food intake (nectar and pollen) and contact with mud of *Osmia cornuta*

		A	B	C	D
#	Products\Categories of bees	Female adult (a: daily exposure; b: nesting period exposure)	Male adult	Female larva (a: daily exposure; b: development period exposure)	Male larva (a: daily exposure; b: development period exposure)

[8]= weight for weight

1	Pollen from provision*/bee bread (oral exposure)	NR	NR	a: 16.3mg/d b: 488mg/30d	a: 9.54mg/d b: 286mg/30d
2	Nectar from provision*/stored in the hive (oral exposure)	a: >18-77mg/d b: >360-1540mg	NR	a: 1.8mg/d b: 54mg/30d	a: 1.07mg/d b: 32mg/30d
3	Pollen from flowers (contact exposure)	a: 128mg/d b: 2.4-4.8 g	NR	NR	NR
4	Sugar from nectar (oral exposure)	a: 18-77mg/d b: 360-1540mg	Unknown	NR	NR
5	Pollen from flowers (oral exposure)	Unknown	NR	NR	NR
6	Mud (contact exposure)	a: 200-400mg/d b: 2.2-4.4g	NR	Unknown	Unknown

(NR): not relevant; (d): day; (*) Considering 10:1 the ratio pollen: nectar in the provision

Table 3.5: Food intake (nectar and pollen) and contact with leaves of *Megachile rotundata*

		A	B	C	D
#	Products\Categories of bees	Female adult (a: daily exposure; b: nesting period exposure)	Male adult	Female larva (a: daily exposure; b: development period exposure)	Male larva (a: daily exposure; b: development period exposure)
1	Pollen from provision*/bee bread (oral exposure)	NR	NR	a: 3.1mg/d b: 31mg/10d	a: 2.6mg/d b: 26mg
2	Nectar from provision*/stored in the hive (oral exposure)	a: >44-66mg/d b: >1800-2700mg	NR	a: 6.2mg/d b: 62mg/10d	a: 5.2mg/d b: 52mg/10d
3	Pollen from flowers (contact exposure)	a: 48-96mg/d b: 1.3-5.4 g	NR	NR	NR
4	Sugar from nectar (oral exposure)	a: 44-66mg/d b: 1800-2700mg	Unknown	NR	NR
5	Pollen from flowers (oral exposure)	Unknown	NR	NR	NR
6	Leave (contact exposure)	a: 172.5mg/d b: 4.9g	NR	Unknown	Unknown

(NR): not relevant; (d): day; (*) considering 1:2 the ratio pollen: nectar in the provision

Further details on the estimated food intake of solitary bees, or food and nesting materials they come into contact with, are described in Appendix F.

3.3.4 Pesticides residues

3.3.4.1 Residues in nectar, pollen, comb, wax and honey bees

Quantifications of residues in bee matrices have been conducted by gathering data coming from (i) scientific literature (Appendix G, Table 1), (ii) the DAR for some substances (Appendix G, Tables 2-5) and (iii) from a national monitoring network in Germany (Appendix G, Tables 6-11).

Data from Appendix G (Tables 1 and 6-11) are not exhaustive, but they show the variability found in the amount of residues contained in bee matrices in Europe. If more data was collected from other countries in the EU, the variability would probably still increase.

The amount of residues detected in bee matrices relies on the levels of the limits of detection (LOD) and quantification (LOQ) used. In this respect, it is important to note that multi-residual analytical methods have higher LOD and LOQ than methods consisting of detecting and quantifying one specific compound.

When compounds present high toxicity levels for bees (either for acute, chronic or sub-lethal toxicity), LOD and LOQ need to be low enough to detect and quantify such compounds. In the case of highly toxic compounds, multi-residual analytical methods which do not present such low LOD and LOQ need to be replaced by more specific detection methods.

The results presented in Appendix G (Tables 1 and 2-5) show that bee matrices are exposed to several pesticide residues and sometimes in high amounts. These compounds belong to all categories of pesticides (insecticides, acaricides, fungicides and herbicides).

3.3.4.2 Residues in nectar and pollen following spray applications and systemic seed treatments

Spray applications on flowering crops or honey dew cause a contamination of nectar and pollen. Residues may also be translocated in nectar and pollen from spray applications and systemic seed and soil treatments (SSST). For residues in nectar, pollen and honey, data is only available for a limited number of substances. The concentration in nectar and pollen can be used to predict exposure to both foraging bees and bees of other casts in the hive, including larvae. Further data on residues in nectar and pollen following spray applications and systemic seed treatments are described in Appendix G (Tables 7-11).

In semi-field and field-trials residue analyses from forager nectar and pollen could easily be obtained. It is recommended that for substances of concern, e.g. toxic insecticides, these data should be provided for a worst case crop. It may not be possible to conduct studies on residues in flowers of every crop, thus realistic worst case model crops should be identified and methods developed to predict worst case residue levels in relevant matrices (e.g. bees, pollen, nectar) to compare with laboratory toxicity data, such as acute or chronic LD50 values for adult bees, and acute and chronic dietary toxicity data for adult bees and larvae for both foliar-applied products and systemic compounds. Further research activities on developing such models seem necessary.

3.3.4.3 Residues in guttation droplets

In the last few years, attention has been posed to guttation of systemic pesticides as a possible exposure pathway for water collecting bees. Measurements of high residue levels of some intrinsically highly toxic, systemic insecticides in guttation droplets from different crops were reported by different researchers (Girolami et al., 2009; Schenke, 2010) and triggered research activities on the risks for bees in 2009. Studies have since been conducted on the environmental conditions and factors favoring guttation, the occurrence of guttation in different crops, the frequency of guttation events and residue measurements in guttation droplets in different crops with different active ingredients in different growth stages (Pistorius et al., 2011a). Different approaches of studies with bees in lower and higher tier tests were set up to gain clarification about possible effects on bees and how this concern would need to be specifically addressed in the risk assessment for bees. So far, guttation has not been specifically addressed in risk assessment (Pistorius et al., 2011b). Meanwhile, there is more information available from laboratory studies, semi-field and field studies as well as post-registration monitoring.

The knowledge of the content of active substances in guttation fluid is important to enable the assessment of the possible risk for honey bees in case they pick up these droplets.

Different crops vary in the intensity and frequency of guttation events (Joachimsmeier et al., 2011) and the amount of residues depends on the properties of the active substance, the amount of active per seed and other factors. While some crops show guttation only in younger growth stages, some may show guttation up to inflorescence. Highest residues were found in all crops at younger growth stages,

showing decline with increasing plant age and growth stage (Schenke 2010, 2011; Reetz et al., 2011). Further data on residues in guttation droplets are described in Appendix E.

3.3.4.4 Residues in propolis

Propolis is collected by bees as resin from trees, e.g. buds, primarily poplars and pine trees, and is used within the hives to block small gaps and as a defense at the hive entrance against ants (etc.). It is also used as an anti-bacterial antifungal agent within the hive. Foragers collect the resin in their pollen baskets to return it to the hive. The chemical composition of propolis varies between sources but is a mixture of resins, terpenes and volatiles. Due to the range of sources of propolis and storage within the hive, propolis collected for human use (e.g. due to its antibacterial and antifungal properties) can contain a range of contaminants.

There are only a small number of reports of trace residues of pesticides present in propolis collected from colonies and propolis tinctures prepared from this. Contaminants include organophosphate pesticides (coumaphos, chlorpyrifos, ethion in Uruguay (Pérez-Parada et al., 2011); dichlorvos, diazinon, malathion, methyl parathion and coumaphos in Mexico (Acosta-Tejada et al., 2011)); pyrethroid residues in Brazil (dos Santos et al., 2008) and varroacide residues in Croatia (Cvek et al., 2009). Bogdanov (2006) reviewed pesticide residues in bee products and concluded that the major contaminants of concern in propolis are lead and persistent lipophilic acaracides, i.e. varroacides applied within the hive.

3.4 Potential exposure routes via non-plant routes

The so-called “non-plant routes of exposure” correspond to all routes that are not related to plant products (i.e. nectar, pollen, guttation water, honey dew etc.). These are described in Appendices I to K. These appendices are mainly limited to exposure in fields where the plant protection product is applied, and to hives located at the edge of these fields.

The exposure assessment of all types of organisms (including that of bees) depends on the application method of the plant protection product (e.g. EFSA, 2012b) and the cropping system. Therefore the description given in the appendices is structured on the basis of the application method, i.e. for spray applications (Appendix I), seed treatments (Appendix J) and injection of soil fumigants (Appendix K).

The most important application methods are (i) spraying of a liquid (emulsions, suspensions or solutions), (ii) seed treatments and distribution of granules and (iii) injection of soil fumigants. Considering non-plant routes of exposure, bees can be exposed to molecules of plant protection products (i) in the non-aqueous phase of spray droplets (in the spray cloud or on plant and soil surfaces shortly after application) of the spray liquid (aqueous emulsions), (ii) sorbed to solid particles suspended in droplets (in the spray cloud or on plant and soil surfaces shortly after application) of the spray liquid (e.g. wettable powders, suspension concentrates resulting in aqueous suspensions), (iii) dissolved in spray droplets (in the spray cloud or on plant and soil surfaces shortly after application) of the spray liquid, (iv) sorbed to solid particles originating from the applied product in the air (dusts: aerosols), (v) in the gas phase in the air or in the soil, (vi) in the liquid phase in the soil and (vii) in water on the soil surface (puddles) and in ditches, streams or ponds. Plant protection products degrade on plant and soil surfaces and in soil and surface water and exposure to the resulting metabolites may of course also occur. The methods for assessing exposure to plant protection product molecules still present in the applied formulated product differ strongly from those for assessing exposure to such molecules present in soil, water and air after the product has become separated from the formulation. Therefore the Appendices F-H dealing with the different application methods deal firstly with exposure to molecules still present in the applied formulated product and secondly with molecules present in soil, water and air.

3.5 Assessment of bees exposure to pesticides

The potential and relevant exposures (by oral, contact and inhalation routes) of bees to sprayed, non sprayed (systemic and non-systemic) insecticides, to dusts from systemic and non-systemic insecticides and to soil fumigation were evaluated by expert judgement. First, the Working Group estimated the presence or absence of exposure and then, a smaller group of experts with a knowledge of the biology of bees and the fate of pesticides in bee products ranked the identified routes of exposure according to (i) the known amounts of food intake, products in contact with or inhaled by bees (see section 3.3.1-3.3.3and Appendices D-F) and (ii) known levels of residues in these matrices (see section 3.3.4 and Appendices G-K). The final result was then presented to the whole Working Group for a final validation.

The ranking ranged from 1 to 4 as follows:

- 0: no route of exposure
- 1: potential route of exposure but negligible
- 2: relevant route of exposure but minor
- 3: relevant route of exposure and high
- 4: relevant route of exposure and very high.
- 1-4?: in situations where no judgement could be made because of incomplete evidence (e.g. data available for i), but where data highlighted a relevant exposure potentially above 1.

The ranking was made both horizontally (across categories of bees for one given product) and vertically (across products for one single category of bee) within the table on exposures (Table 3.1). For each category of bees, we considered the worst case scenario at any given time (e.g. for queens and drones, the worst case was envisaged during mating flights). When potential multiple and relatively high exposures were detected, they were highlighted.

The details of these assessments are presented in the appendices for honey bees (Appendix L), bumble bees (Appendix M) and solitary bees (Appendix N).

Based on the ranking made (see Appendix Tables L1-6, M1 and N1 for more details), the most exposed categories of bees and the most relevant routes of exposures could be highlighted by selecting the highest scores (“4”) and by taking into account the multiple exposures that could occur at any given time.

3.5.1 Honey bees

For honey bees, the highest scores are shown in Table 3.6 for oral exposure and in Table 3.7 for exposures by contact and inhalation.

Oral exposures were considered highest for foragers with nectar and honey dew (whether coming from plants or the hive), winter bees with honey (whether coming from nectar or honey dew) and larvae with pollen bee bread (Table 3.6). When considering potential multiple exposures, foragers were the most exposed followed by winter bees and nurses.

Given the lack of data on the type of water used by foragers (whether coming from puddles, surface, leaves and/or axils), it was not possible to conclude whether foragers are highly exposed through this mode of exposure. However, given the uncertainty and high levels of residues found in water, this category of bee and exposure should be taken into consideration.

Table 3.6: Oral exposure from residues and dusts from sprayed and non-sprayed, systemic and non-systemic insecticides

	Foragers	Wax producing bees	Nurse bees and brood attending bees	Winter bees	Mating drones	Mating queens	Swarms	Larvae
Oral exposure								
Nectar from plant	4							
Nectar in-hive	4		(3)*					
Honey from nectar				4				
Residues in pollen and bee bread			(3)*					4
Honeydew	4							
Honeydew in-hive	4							
Honey from honey dew				4				
Water (leaf axils)[d]	1-4?							
Water (puddles in field)[d]	1-4?							
Water (surface water)[d]	1-4?							

(*) multiple exposure

Exposures by contact were considered highest for larvae with wax and exposures by inhalation were considered maximal for foragers, drones, queens and swarms with interception of droplets and vapour in/out field (Table 3.7). Given the lack of data on the amount of propolis in contact with larvae and the amount and fate of water inhaled by in-hive bees, it was not possible to conclude regarding these categories of bees (i.e. larvae, wax producing bees, nurse and brood attending bees).

Table 3.7: Exposure by contact with and inhalation from soil fumigant

	Foragers	Wax producing bees	Nurse bees and brood attending bees	Winter bees	Mating drones	Mating queens	Swarms	Larvae
Contact/Dermal exposure								
Propolis								1-4?
Wax contaminated via pollen and nectar								4
Interception of droplets (direct overspray)	4				4	4	4	
Exposure by inhalation								
Vapour in/out field	4				4	4	4	
Vapour within the hive		1-4?	1-4?	1-4?				1-4?

3.5.2 Bumble bees

Oral exposures were considered highest for workers and queens with nectar and honey dew and larvae with pollen carried in the nest (Table 3.8). Because most species nest in the soil, exposures by contact and by inhalation (from soil fumigant) were considered highest for all categories of bees. Exposures by contact and inhalation were considered maximal for workers, drones and queens with interception of droplets and vapour in/out field (Table 3.8).

Table 3.8: Exposure by oral, contact and inhalation from pesticides in bumble bees

Source of exposure	Workers	Drones	Queens	Larvae
Oral exposure				
Nectar (from plant)	4		4	
Pollen/bee bread				4
Honey dew	4		4	
Contact/Dermal exposure				
Exposure to Soil	4	4	4	4
Interception of droplets (direct overspray)	4	4	4	
Exposure by inhalation: SOIL FUMIGANT				
Vapor in/out field	4	4	4	
Vapor in the soil	4	4	4	4

3.5.3 Solitary bees

In solitary bees, oral exposures were considered highest for adult females with nectar and honey dew and larvae with pollen in the provision (Table 3.9). Adult females and larvae showed maximum exposure by contact with soil, foliar residues or other nesting material. In fact, many solitary bees make nests in the soil or use mud and plant products as nesting material. Exposures by contact and inhalation were considered maximal for adult females and males with interception of droplets and vapour in/out field (Table 3.9). Exposure by inhalation (soil fumigant) was considered maximum for nesting females and larvae in species nesting in the soil.

Table 3.9: Exposure by oral, contact and inhalation from pesticides in solitary bees.

Source of exposure	Adult-Female	Adult-Male	Larvae
Oral exposure			
Nectar (from plant)	4		
Pollen/bee bread			4
Honeydew	4		
Contact/Dermal exposure			
Nesting Material propolis/resins	4		4
Exposure to Soil	4		4
Foliar Residues (contact)	4		4
Interception of droplets (direct overspray)	4	4	
Exposure by inhalation: SOIL FUMIGANT			
Vapor in/out field	4	4	
Vapor in the soil	4		4

4 CHAPTER 4: TOXICITY OF PESTICIDES IN BEES AFTER REPEATED EXPOSURE AT SUB-LETHAL DOSES

4.1 Summary

Sub-lethal doses can be defined as a fraction of the LD50 (the amount of a solid or liquid material that it takes to kill 50% of test animals in one dose) and are often an order of magnitude below such lethal doses (below LD50/10). A comprehensive review of the literature was performed and reports sub-lethal toxicological effects of pesticides in honey bees, bumble bees and solitary bees. The overview of the available studies on such sub-lethal doses and long-term effects of pesticides on bees (*Apis mellifera*, social non-*Apis* and solitary bees), mostly available from laboratory studies, highlighted gaps in knowledge and future research needs. From this body of evidence, it is recommended that more toxicological studies are performed in bees for a wider range of pesticides on both adults and larvae using sub-lethal endpoints, including biochemical, neurophysiological effects, effects on reproduction and life - span (fecundity and longevity) and measurement of long-term effects. Since most studies are available for the oral route of exposure, future studies should also be designed for the contact and inhalation route on the different honey bee subspecies (since most studies do not specify the subspecies tested). Additionally, these studies should also be performed in field conditions since sub-lethal effects have mostly been measured in laboratory conditions. With specific regard to non-*Apis* bees, very few studies are available in literature and only for a very limited number of species (solitary bees and bumble bees). These studies considered endpoints such as fecundity (e.g. drones production in *Bombus* and cell production rate in solitary bees), larvae mortality rate, adult longevity and foraging behaviour. The use of micro-colonies in bumble bees appears to be well-suited to measure lethal and sub-lethal effects of pesticides with low doses and long-term effects.

Furthermore, because of the specific toxicokinetic profile of bees compared with other insects, it is recognised that toxicokinetic data can provide useful information on the potential bioaccumulation of a pesticide after repeat exposure which, in some cases, could have adverse effects that may be either more marked compared with their short-term effects or irreversible. Overall, the integration of toxicokinetic knowledge and sub-lethal dose effects generated from laboratory and field studies in the hazard identification and hazard characterisation of pesticides in *Apis* and *non-Apis* bees can provide a better understanding of short-term and long-term effects. It is therefore concluded that the conventional regulatory tests based on acute toxicity (48 to 96 h) are likely to be unsuitable to assess the risks of long-term exposures to pesticides. A number of options are given to improve the current testing protocols to detect bioaccumulation of single active substances in honey bees after repeated exposure and the potential consequence on toxicity

A testing protocol and mathematic model, based on Haber's rule as a conservative approach, have been developed to investigate the potential bioaccumulation of pesticides after repeated exposure in honey bees and applied to mortality data. When applied to data from standard dose-mortality trials, the testing protocol and model demonstrate that pesticides will indeed vary in their capacity to bioaccumulate and generate adverse effects after repeated exposure. However, a number of assumptions inherent to the model raise uncertainties regarding the irreversibility of the effect and receptor kinetics, the model does not take into account the consequence of metabolism (first order, second order or n-order kinetics) and the potential for saturation (increase or decrease toxicity), the non-linearity of the toxicokinetics, and/or of the toxic effects. Indeed, such a protocol and model would need further validation in the laboratory and to be tested for sub-lethal endpoints in adult and bee larvae. Finally, combining basic toxicokinetic data for an active substance and its metabolites, such as the half life, will also provide more precise quantitative estimates on the potential for bioaccumulation and in the case of potential persistence of the active ingredient, half life of the parent compound and its metabolites should be determined in larvae, newly emerged bees and foragers.

4.2 Overview of studies dealing with sub-lethal doses and long-term repeat dose effects of pesticides on bees

Sub-lethal doses can be defined as a fraction of the LD50 (the amount of a solid or liquid material that it takes to kill 50% of test animals in one dose and are often an order of magnitude below such lethal doses (below LD50/10). A comprehensive review of the literature has been performed and reports sub-lethal toxicological effects of pesticides in honey bees, bumble bees and solitary bees. Such sub-lethal effects of pesticides on bees have previously been extensively reviewed by a number of authors (Haynes, 1988; Thompson, 2003; Desneux et al., 2007; Decourtye and Devillers, 2010; Blacquière et al., 2012). Collectively, the current review reports evidence for sub-lethal effects of pesticides on all major species of *Apis* and *non-Apis* bees (honey bees, *Apis mellifera*, see Appendix A; social non-*Apis* such as bumble bees, *Bombus spp.* and solitary bees, see Appendix Q). Such sub-lethal effects have been reported for a variety of endpoints including biochemical, physiological and behaviour endpoints (cholinesterase activity, survival, development, longevity, locomotion or mobility, navigation or orientation, feeding behaviour and learning performance).

Appendices P and Q summarise the design for each individual study including the substance, bee species, doses tested, type of study (laboratory/field), study duration, life stage, endpoints measured and the main results. In this review, no attempt was made to assess each individual study in details.

Potential synergistic effects of pesticide mixtures on bees and the interaction between low doses of pesticides and other stressors, such as diseases, are discussed in Chapter 7.

4.2.1 Honey bees

Overall, there is a larger number of studies reporting sub-lethal acute effects compared with long-term effects. Three studies reported either no effect of imidacloprid and its metabolites (Schmuck, 2004) and of clothianidin (Cutler and Scott-Dupree, 2007) or a positive effect of imidacloprid (Stadler et al., 2003) on colonies' development.

When classifying studies according to the endpoint measured, it appears that the endpoints for most studies were survival/mortality, development and learning whereas fecundity, longevity and biochemical/neurophysicological effects were the least studied.

In terms of pesticide classes, studies using insecticides, varroacides and fungicides were available from literature, however, studies using insecticides and particularly imidacloprid and fipronil were the most represented. For other pesticides, few or single studies were available.

Most studies were conducted on *A. mellifera* spp., with the exception of a few studies conducted on Africanised *A. mellifera*, *A. cerana indica, A. m ligustica* and *A. m. carnica*. However, a number of studies referred to honey bees with no specification of the subspecies. Toxicity was measured for the oral route of exposure in adult bees for the majority of studies, with only two studies available for the injection route (measuring biochemistry/neurophysiology and foraging). Amongst the few studies which assessed brood exposure, only one investigated contact exposure.

Appendix A summarises the toxicological studies in honey bees including sub-lethal endpoints such as biochemical, neurophysiological effects, fecundity and longevity, and when available long-term effects.

4.2.2 Bumble Bees

Toxicity studies, reporting from literature sub-lethal effects on social non-*Apis* bees include mostly one species of bumble bees (*Bombus terrestris*) with the exception of two studies available for *Bombus impatiens* and one on *Bombus occidentalis.* These studies have mostly been performed for the oral route in laboratory conditions using single adult bee or microcolonies and are summarised in Appendix Q for

insecticides, varroacides and fungicides. It is concluded that the use of microcolonies appears to be well-suited to measure lethal and sub-lethal effects of systemic insecticides on bumble bees as well as insecticidal proteins expressed in transgenic plants. In several studies, the drone production (as a measure of fecundity) was measured in laboratory conditions using microcolonies.

4.2.3 Solitary bees

Few authors have investigated the chronic effects of pesticides on solitary bees at low (sub-lethal) doses. Reported studies involved only five species of solitary bees reared as crop pollinators: *Nomia melanderi*, *Megachile rotundata*, *Osmia cornuta*, *Osmia bicornis*, *Osmia lignaria*. Tests were performed on adults and larvae in laboratory, semi-field and field conditions. In comparison with the data available for honey bees, more studies investigated effects of pesticides on fecundity and development, but fewer studies were carried out to investigate behavioural effects. In solitary bees, the effects of pesticides on fecundity of the nesting females were studied in cage or semi-field conditions (see Appendix Q for more details). Because most studies investigating low (sub-lethal) dose effects were carried out separately on single species, inter-species differences between solitary bees should be quantified to assess their relative sensitivity to the toxicity of pesticides.

4.3 Bioaccumulation of pesticides in honey bees and repeat dose effects

Recently, concern has been raised over the repeat dose or long-term exposure of bees to pesticide residues that may bioaccumulate. Such pesticide residues may have repeat dose effects that may potentially have more dramatic health effects compared with their short-term effects (Tennekes, 2010; Tennekes and Sanchez-Bayo, 2011).

The rationale for assessing the bioaccumulation potential of single active substances and its consequence on toxicity after repeat dose exposure in bees is based on the risk assessment paradigm: hazard identification and characterisation, exposure assessment and risk characterisation. In this context, the hazard identification and characterisation of pesticides involves an understanding of the fate of the compound in the bee (toxicokinetics) and the dose response of the compound to characterise the toxicity (toxicodynamics). Ideally, once these toxicokinetic and toxicodynamic dimensions are characterised, the risk can be identified through a comparison of toxicity with environmentally-relevant exposure.

In terms of toxicokinetics, 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 i.e cytochrome P-450, glutathione-S-transferases, carboxyesterases (see Chapter 7 for more specific details). Such a metabolic and elimination capacity in honey bees makes them potentially sensitive to compounds with a bioaccumulation potential and an understanding of the overall elimination of the compound, such as the half life of the parent compound and its metabolites as a marker of potential persistence, together with the consequence of metabolism i.e. whether the toxicant is the metabolite or the parent compound, is required.

In terms of toxicodynamics, a characterisation of the toxicity, whether such toxicity is irreversible, persistent or delayed and its dose–response in relation to time of exposure is necessary. The review of sub-lethal effects in *Apis* and *non-Apis* bees described at the beginning of this chapter demonstrates that the analysis of the dose response should not only be confined to mortality and adult bees but should also integrate sub-lethal effects and data on larvae respectively. Again one of the difficulties is that most of the data from most sub-lethal studies are limited and generated from laboratory studies on individuals, not populations. Hence, the relevance of sub-lethal studies from the laboratory to semi-field studies and field studies still remains to be explored and more research is needed in this area.

When dealing with bioaccumulative potential, it may be assumed on a case by case basis that the consequential toxicity may also have a toxicokinetic basis (long half life of the compound or its metabolites) and that the toxic effects on a small proportion of the population combined with repeated exposure may lead to an aggravation of the effect with time.

Whilst the bioaccumulative nature of certain compounds has been contested (Maus and Nauen, 2010; Tennekes, 2010b), biological persistence nevertheless presents a potential risk to bees that should be assessed. However, the conventional regulatory tests are likely to be unsuited to assess the risks of long-term exposures because they are based on short-term measurements (48 to 96 h), and may fail to detect the true potential for long-term effects. Recently, Haber's law has been proposed to assess the potential for cumulative effects of pesticides in honey bees. Haber's law is a simple linear relationship and has been applied in the past in human risk assessment of chemicals. However, its use has been a matter of debate since it assumes irreversibility of the effect and may not take into consideration the consequence of metabolism (increase or decrease toxicity), non-linearity of the toxicokinetics, receptor kinetics and/or of the toxic effects (Druckrey and Küpfmüller, 1948; Tennekes, 2010a; Tennekes and Sanchez-Bayo, 2011; Maus and Nauen, 2010). Recently, more sophisticated models have been proposed and include a generalised Haber's law for exponential concentration decline applied to aquatic pesticide ecotoxicity using first order kinetics (Bogen and Reiss, 2012); Dynamic Energy Budgets (DEB) to model receptor kinetics in the analysis of survival data for organophosphorous pesticides (Jager and Kooijman, 2005). Since toxicokinetic data and data reporting sub-lethal effects of pesticides in bees are scarce, the use of mortality data and Haber's law is illustrated as a first step to prioritise pesticides which may have cumulative effects in bees. However, it can be foreseen that in the future refined models and experiments can be developed when more mechanistic and quantitative toxicological data on pesticides in bees becomes available.

4.3.1 Haber's law, bioaccumulation and repeated dose effects of pesticides in honey bees

Haber's law states that the severity of an adverse effect on an organism's health depends on the total exposure to a toxic compound (Gaylor, 2000). Haber's law is a model where the total exposure is defined as the concentration of the compound at the target site or tissue (C) and expressed in molarity units (M) multiplied by the duration of the exposure (t) expressed in hours. The total exposure (k) is given by the product ($C \times t$) and is expressed in molar hours (Eq.1). k assumes that the total effect is proportional to the total exposure.

$$C \times t = k \qquad \text{Eq.1}$$

When taken as a worst case scenario, Haber's law aims to extrapolate the results of short-term tests to untested longer exposures to lower doses but with equivalent molar hours (Eq 1). In many cases, the concentration of the toxin at the target site (internal dose or concentration) is unknown and can only be quantified in an environmental concentration, which is often the concentration of the toxin in the organism's surrounding medium or in its diet (environmental concentration or external dose). This environmental concentration can be then used as a proxy for C and denoted by C_P.

For a dietary compound, C_P constitutes a worst case scenario since it assumes a 100% absorption of the compound i.e. when extrapolating from Eq 1, C_P is a proxy for C if an individual's internal concentration is both proportional to the compound's dietary concentration and constant despite continuous ingestion. However, for a dietary compound that may bioaccumulate, simple extrapolations may provide erroneous estimates since the value of C at the site of action increases with time even when the dietary concentration, C_P, is constant (Figure 4.1). In this case, using C_P instead of C would mean that extrapolations from short-term exposures using Haber's law (Eq 1) may underestimate long-term effects.

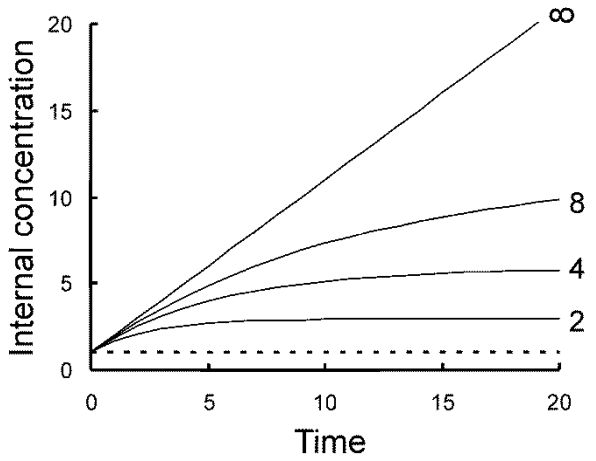

Figure 4.1: Internal concentration in relation to time for an individual ingesting one unit of dietary toxin per unit of time. The graph illustrates the relationship for five different toxins with different biological half-lives. The dashed line represents a toxin that is eliminated by the end of each time unit (t_{50} below 2 time units) whereas the other four examples illustrate compounds with longer half lives (t_{50} = 2, 4, 8 and ∞ time units).

An individual's total exposure in molar units-hours is given by the product of the individual's internal concentration of the toxicant and the duration of exposure. For example, if an individual receives a daily unit dose in its diet, which has a concentration of C_P = 1 M, and the toxicant is assumed to be eliminated completely by the end of each day, the estimated total exposure over 20 days is 20 molar days, or 480 molar hours. However total exposure may be much greater for bioaccumulative toxins (i.e. toxins with high values of t_{50}), particularly as the duration of the exposure increases (Figure 4.1). For example, if the toxicant bioaccumulates completely ($t_{50} = \infty$), the total exposure is 200 molar days (Figure 4.1).

In order to detect, potential bioaccumulation of pesticides in bees from dietary exposure and repeated dose effects, an examination was carried out to see whether equivalent exposures (i.e. specified in molar-hours) may cause equivalent effects (based on mortality) when the exposures are calculated based on the dietary concentration, C_P. Specifically, an investigation was made to see whether the same effect on mortality may be obtained by exposure to concentration C_P after t days and by concentration C_P/α after αt days, where $\alpha>1$ so that $(\alpha C_P, t/\alpha)$ is the high concentration-short duration exposure. If C_P is a fair proxy for C because the toxin is not persistent, the graphical relationship between the magnitudes of the effects observed at (C_P, t) and at $(C_P\alpha, t/\alpha)$ should be a straight line through the origin with a slope of a positive one. If instead the toxin is persistent, then the low-dose long-duration combinations (C_P, t) will produce disproportionately high toxin loads (Figure 4.1) that cause correspondingly severe effects and the relationship between the effects at C_P/α and C_P will be displaced from the line with a slope of a positive one.

In order to prioritise pesticides which may potentially bioaccumulate, the methodology can be tested in the laboratory using standard toxicity tests, measuring daily mortality over time in cages of 20 individual bees with each compound delivered orally in the feeder syrup.

Under Haber's law, any equivalent product of C and t yields an equivalent total exposure, denoted k, and therefore an equivalent effect, i.e.

$$C \times t = k \qquad \text{Eq.1}$$

and so for a fixed value of k, we require

$$C/\alpha \times \alpha t = C\, t \qquad \text{Eq.2}$$

Taking the theoretical maximum span of an experiment of 10 days and a minimum α value of 0.1, it is assumed that the shortest feasible duration to measure mortality is one day and that measurements are performed daily so that in practice, the shortest feasible duration is two days ($\alpha \geq 0.2$).

In order to measure the range of mortality frequency, it would be necessary for the doses to range across approximately two orders of magnitude. As an example, assuming the highest concentration at 100 μg L^{-1}, Table 4.1 illustrates an experiment with seven doses that would generate a dose-response over two orders of magnitude :

Table 4.1: Dose-exposure relationships in a hypothetical experiment to test for bioaccumulation and repeat dose effects.

DOSE (μg L^{-1})	DAYS
100	5
50	10
20	10
10	10
4	10
2	10
0.8	10

The dose-exposure time relationship designed to test for bioaccumulation and repeat dose effects are arranged for $\alpha = 0.2$ (which compares cumulative mortality between appropriate doses on days $t = 2$ and $t = 10$) and $\alpha \geq 0.5$ (which compares days 5 and 10).

Table 4.2: Dose-exposure time relationship in a hypothetical experiment to test for bioaccumulation and repeat dose effects (doses given in Table 4.1). Dose-exposure time that share a line produce equivalent exposures (or dose-duration products, Ct)

	Exposure A			Exposure B	
Ct	**dose (*C*)**	**duration (*t*)**		**dose (*C*)**	**duration (*t*)**
200	100	2		20	10
40	20	2		4	10
8	4	2		0.8	10
500	100	5		50	10
100	20	5		10	10
20	4	5		2	10

Theoretical mortality-exposure time relationships (i.e. cumulative mortality) at each dose are shown below.

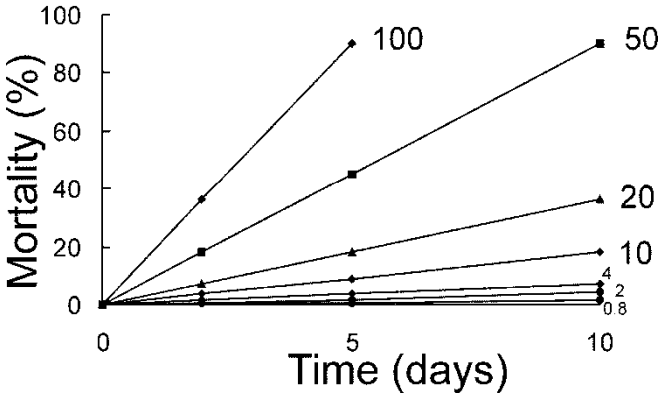

Figure 4.2: Cumulative mortality over time at seven different doses (100, 50, 20, 10, 4, 2 and 0.8 µg L^{-1} in a theoretical experiment (design described in Table 4.2).

These relationships produce the dataset below (Table 4.3).

Exposure A			Exposure B		
C	***t***	**mortality %**	***C***	***t***	**mortality %**
100	2	36	20	10	36
20	2	7.2	4	10	7.2
4	2	1.44	0.8	10	1.8
100	5	90	50	10	90
20	5	18	10	10	18
4	5	3.6	2	10	4.5

Table 4.3: Percentage mortality among bees in a theoretical dose-exposure time relationship experiment to test for bioaccumulation and repeat dose effects (Data from Figure 4.2).

These data support the proposed graphical test for bioaccumulation and repeated dose effects, which indicates whether equivalent exposure (calculated on dietary concentrations) produces equivalent mortality, as follows.

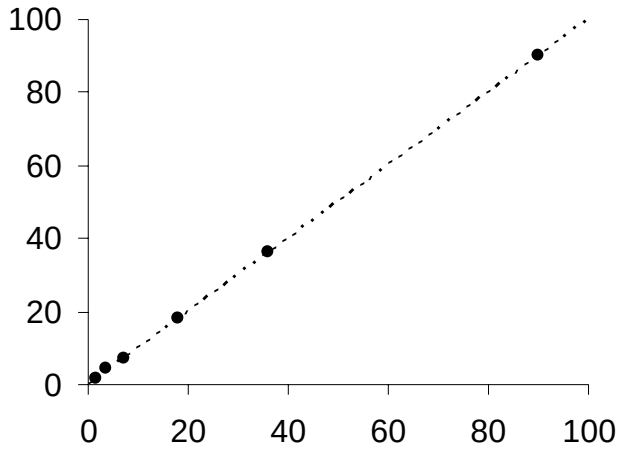

Figure 4.3: Test for equivalence of effect in equivalent exposures produced by different dose-duration combinations in a theoretical experiment (Data from Table 4.3).

These theoretical data show that different dose-exposure time relationships of equivalent exposure produce equivalent mortality even when exposure is calculated based on dietary concentrations, rather than internal concentration. In this case, it would be concluded that there is no indication that the compound is bioaccumulative.

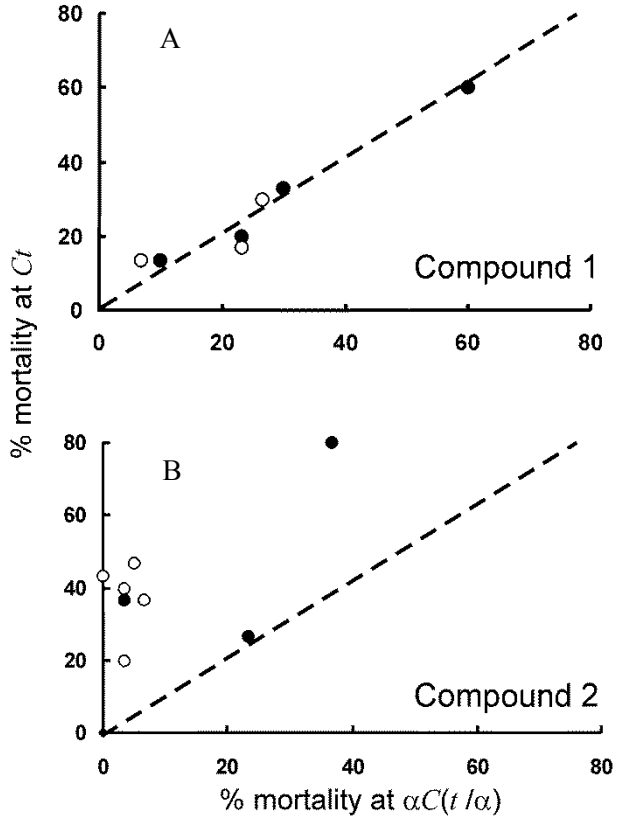

Figure 4.4: Graphical test for persistence of two pesticides (A compound 1 and B compound 2) in dietary exposures to honey bees. Each panel shows the relationship between levels of mortality (%) observed at doses C after t days (y-axis) and dose C/α after αt days (x-axis) for $\alpha = 2$ (closed symbols; combinations from C = 4000, 2000, 1000, 500, 250 and t = 5 days, 10 days) and $\alpha = 4$ (open symbols; combinations from C = 4000, 2000, 1000 and t = 2 days, 8 days). Data from Defra (2007).

In experiments on toxicity in honey bees (Defra, unpublished), dietary exposures to compound 1 produce approximately equivalent levels of mortality at equivalent exposures independent of timescale up to 10 days (Figure 4.4 A), which suggests that the toxicant is not persistent. In contrast, dietary exposures to compound 2 produce disproportionately high levels of mortality at longer timescales (Figure 4.4 B), which suggests that the toxicant may either bioaccumulate from a toxicokinetic point of view or cause repeat dose effects or irreversible adverse effects from the toxicodynamic point of view.

Overall, this method based on Haber ‘s rule may provide a potential simple prioritisation tool to assess bioaccumulation and repeated dose effects of single pesticides using mortality data. However, a number of assumptions inherent to the model raise uncertainties regarding the irreversibility of the effect and receptor kinetics. Furthermore, the model does not take into account the consequence of metabolism (first order, second order or n-order kinetics) and the potential for saturation (increase or decrease toxicity), the non-linearity of the toxicokinetics, and/or of the toxic effects. Additionally, laboratory tests would need to investigate whether Haber’s Law can be used as a potential prioritisation tool for sub-lethal effects and relate those effects to semi-field and field studies for adult and bee larvae. Finally, combining basic toxicokinetic data for an active substance and its metabolites will also provide more precise quantitative estimates on the potential for bioaccumulation and repeat dose effects. In the case of bioaccumulation of

the active ingredient, toxicokinetics should be assessed in larvae, newly emerged bees and foragers (as the metabolising enzymes of honey bees develop over time (Smirle and Winston, 1987; Papadopoulos et al., 2004) to determine the half life of the active ingredient and any toxic metabolites as a basis of this observation.

5 CHAPTER 5: THE EVALUATION OF THE EXISTING TEST PROTOCOLS FOR HONEY BEES, SOCIAL NON-*APIS* (BUMBLE BEE) AND SOLITARY BEES

Following the Regulation 1107/2009 (Annex II, 3.8.3.) *"An active substance, safener or synergist shall be approved only if it is established following an appropriate risk assessment on the basis of Community or internationally agreed test guidelines, that the use under the proposed conditions of use of plant protection products containing this active substance, safener or synergist: will result in a negligible exposure of honey bees, or has no unacceptable acute or chronic effects on colony survival and development, taking into account effects on honey bee larvae and honey bee behaviour***."**

This chapter deals specifically with one of the questions of the Mandate from the Commission to EFSA for an *"evaluation of the existing validated tests protocols and the possible need to develop new protocols especially taking into account the bees' exposure to pesticides on nectar and pollen"*.

According to the specific protection goals defined in Chapter 2 of this opinion, the test methods considered have been analysed for their capacity to detect the magnitude of effects and the attributes and temporal scale on the ecological entities have been considered.

5.1 Summary

Several methodologies have been considered to identify the effects of pesticides on bees. They can be conducted in laboratory, semi-field and/or field conditions.

Laboratory tests

At the moment, the EU risk assessment of pesticides on bees includes the determination of oral and contact acute toxicity (LD50) of adult honey bees (*Apis mellifera*) following EPPO guidelines 170 and OECD 213 and 214. Several sources of variation of this parameter have been identified. However, potential effects on non-*Apis* bees have never been evaluated before.

Several exposure routes of pesticides are not evaluated in laboratory conditions: the intermittent and prolonged exposure of adult bees, exposure through inhalation and the exposure of larvae. Likewise, the effects of sub-lethal doses of pesticides are not evaluated in conventional testing for now. Consequently, it is recommended to include in first tier: chronic toxicity tests on adult bees, a laboratory larval toxicity test, and precise observations of sub-lethal effects. Ultimately specific tests aimed at evaluating sub-lethal effects should be adopted in the first tier of testing. Some examples have been identified in this opinion: Proboscis Extension Reflex, homing flight or *Bombus* microcolonies. Several points for further research have been identified.

Semi-field tests

Semi-field testing (cage, tunnel or tent tests) are currently conducted following three test guidelines: the EPPO 170 (4), the OECD 75 brood guidance document (OECD, 2007), and the Oomen et al. (1992) test. Several weaknesses have been identified for each of these guidance documents, e.g. the limited size of crop area, the impossibility to evaluate all the possible exposure routes of the systemic compounds used as seed- and soil-treatments (SSST), the limited potential to extrapolate the findings on larger colony sizes used in field studies or the relatively short timescale (one brood cycle). It is recommended to add the current state of scientific knowledge on a number of issues to the existing guidelines (e.g. more detailed description and categorization of behavioural assessments, investigation of sub-lethal and delayed effects, etc.) and to develop methodologies adapted to pesticides applied as SSST, including potential risks (e.g. for dust drift and guttation). Consideration should be given to extending studies where significant exposure is likely to occur over a long period. Results should be analysed with appropriate statistical methods.

Field tests

Field testing currently follows only one test guideline, the EPPO 170 (4). A precise analysis of this guideline, based on scientific knowledge, showed that it has several major weaknesses (e.g. the small size of the colonies, the very small distance between the hives and the treated field, the very low surface of the test field), leading to uncertainties concerning the real exposure of the honey bees. Even when they are carried out in natural conditions, they may not represent the normal exposure conditions of the bee colonies. Furthermore this guideline is better suited to the assessment of spray products than to that of SSST. For the moment, it does not allow us to assess all the effects of pesticides on the scale of the colony, including all bee categories and relevant long-term effects. It is therefore important and urgent to adapt guidelines to a specific mode of pesticide application and to the relevant exposure routes.

5.2 Laboratory tests

5.2.1 Summary

This section covers tests on two life stages (adult, larva) and two exposure durations (acute, prolonged (>96h)) developed under controlled conditions. A range of laboratory tests on adult honey bees (acute/prolonged exposures) and larval honey bees have been collected and described in this opinion.

Table 5.1 focuses on the methodologies proposed as international or national standards for the determination of the oral and contact acute toxicity on honey bees. From these tests, the LD50 oral and contact toxicity is estimated. This methodology is run systematically for all active ingredients. Different sources of variation of acute toxicity results have been identified. Given the impossibility of testing all possible conditions, the uncertainty in the use of LD50 values needs to be considered for the evaluation of risk.

Table 5.2 proposes methodologies for the evaluation of chronic toxicity tests. None of these methodologies have been validated, nor ring-tested, nor have they been incorporated so far in the risk assessment of pesticides. Following currently existing scientific knowledge, it is recommended to include the evaluation of chronic toxicity in risk assessment on a systematic basis.

Table 5.3 contains the details of the methodology for the determination of larval toxicity data in laboratory conditions. The method for the estimation of acute larval toxicity has undergone ring-testing. These methods, for acute and chronic larval toxicity, have been submitted for OECD validation. It is recommended to include the evaluation of chronic larvae toxicity in risk assessment on a systemic basis.

It is recommended to record all intoxication signs happening during acute and chronic toxicity testing. Additionally, Table 5.4 summarises the details for the development of Proboscis Extension Reflex (PER) test. As happens for chronic toxicity tests, despite having been used in many laboratories, at this moment the Proboscis Extension Reflex lacks ring-testing and validation. It is so far unclear how such a sub-lethal testing should be incorporated in risk assessment. Ultimately specific tests aimed at evaluating sub-lethal effects should be adopted in the first tier of testing

Table 5.5 focuses on the methodologies to run tests in laboratory conditions for bumble bees.

Table 5.6 focuses on the methodologies to run tests in laboratory conditions for solitary bees.

Table 5.1: Adult bees/acute exposure

Test method	Life stage	Exposure route and administration dose	Toxicologic-al endpoint	Duration	Test Conditions	Toxic standard	Control No. of dose and replicates	Stat. evaluation	Comments
OECD 213 Acute oral toxicity test Laboratory	Young worker honey bees	Oral 100-200µl of 50% sucrose solution with a.i. during 6h	Acute LD_{50}	24-48h (extension of test to 72 and 96h if mortality continues to rise after 24 h by more than 10%). Experimentation starts at t0, but observation period is determinate after exposure.	2h starvation 25° C ± 2°C Humidity 50-70% Dark	Dimethoate (24 h LD50 0.1 – 0.30 µg a.i./bee) or parathion	3 control groups of 10 bees each Test valid if average mortality ≤10% in controls after 24-48h 5 dose groups covering the LD50, 3 replicates with 10 bees each	Dose-response curve, median LD50, 95% C.I. (Probit analysis, moving-average, binomial probability) Mortality recorded after 24 and 48h Calculation of the dose consumed	Data available for many substances in EU, standard data requirement
OECD 214 Acute contact toxicity test Laboratory	Young worker honey bees	Contact	Acute LD_{50}	48-96h (extension of test to 96h if mortality continues to rise after 24 h by more than 10%)	anaesthetized with CO_2 or nitrogen 25° C ± 2°C Humidity 50-70%	Dimethoate (24 h LD50 0.1 – 0.30 µg a.i./bee)	3 control group of 10 bees Each test valid if mortality ≤10% after 24-48h 5 dose groups covering the LD50, 3 replicates with 10 bees each	Dose-response curve, median LD50, 95% C.I.	Data available for many substances in EU, standard data requirement
EPA OPPTS 850.3020 Honey bee acute contact toxicity Laboratory	Young worker honey bees, 1-7 d old, from disease-free colonies	Contact	Acute LD_{50}	48 h	25-35°C Humidity 50-80% Dark conditions except during dosing and observations	No toxic standard required	Control included, also solvent control, Mortality <20% at the end of test 5 dose groups ideally from 0 to 100% mortality, 25 bees per dose group (may be divided in	Dose-response curve, median LD50, 95% C.I.	High level of standardisation and reproducibility of results

							replicates)		
EPA OPPTS 850.3030 Honey bee toxicity of residues on foliage Laboratory	Young worker honey bees, 1-7 d old, from disease free colonies	Contact to fresh residues on leaves, after 3h, 8 h and 24 h of sampling	Acute LD50	24 h	No recommend-ations but housing conditions, temperature, humidity and lighting should be reported.	No toxic standard required	Control group included Mortality <20% at the end of test At least the highest recommended field rate, multiples of the maximum rate may be tested if desired 6 replicates with 25 bees each,	Not described (probably NOEC estimate)	Test design similar to extended laboratory tests with non-target arthropods

Table 5.2: Adult bees/prolonged exposure

Test method	Life stage	Exposure route	Endpoint	Duration	Test Conditions	Toxic standard	Control No. of dose and replicates	Stat. evaluation	Comments
Decourtye et al. (2005) Chronic oral toxicity test Laboratory	Young worker honey bees (from 2 to 14-15 days)	Oral (continuous)	NOEC Mortal-ity	11 days	33+-2ºC 40+-10% HR darkness		Control group included 3 dose groups covering the LD50 (in 48h)/20, 3 replicates with 12-20 bees each	Two-by-two X^2 tests with 1 df, critical probability levels 0,0085	Test not ring-tested
Suchail et al. (2001) Chronic oral toxicity tests Laboratory	Worker bees (*A. mellifera*) were captured from honey and pollen combs in a healthy queen-right colony (< 15 days)	Oral (continuous during 10 days. Opaque feeders). 2 hours of fast before administra-tion of fresh syrup	NOEC (Mortal-ity) and LC50	10 days	Bees are anaesthetized with carbon dioxide and kept in cages (10.5 x 7.5 x 11.5 cm) in a temperature-controlled chamber at 25+- 2ºC with 60 +- 10% relative humidity. Bees are fed a 50% sucrose solution *ad libitum*. Darkness	No toxic standard	Control included (mortality <15%) 3 cages, 30 bees/dose/cage, 3 replicates S.a. diluted in a 1% dimethylsulfoxide solution and then diluted 10-fold in the 50% (w/v) feeding sucrose solution. Final concentration of dimethylsulfoxide in the sucrose solutions 0.1% (v/v).	Corrected mortality (Abbot formula)	Test not ring-tested Study aims at differentiating acute toxicity from chronic toxicity
Aliouane et al. (2009) Topic chronic toxicity Laboratory	Newly emerged bees (*A. mellifera*)	Contact (thorax). Bee caught in the cage daily and maintained with an insect forceps,	NOEC (mortal-ity per day regist-ered)	11 days	Cages with 40 individuals, maintained in darkness (40% relative humidity, temperature 33ºC). Pollen and sucrose solution (50% w/v) provided *ad*	No toxic standard	Control received same treatment but only with solvent (acetone)	Kaplan–Meier test to compare control and treated groups.	Test not ring-tested

		while 1 µl of the solution applied using a micropipette with a tip. After disappearance of drop, bee taken to a different cage.			*libitum* for the first week. Bees were then allowed to make a purging flight before returning to their cages. During test period, bees fed with sucrose solution (50% w/v) and water. Feeders changed daily with fresh solutions				
DEFRA (2009) Oral chronic toxicity tests (intermittent) Laboratory	Young workers bees (*A. mellifera*)	Oral (intermittent exposure)	LC50 Mortal-ity	10 days	25 ± 1°C, 65 ± 5% relative humidity Dark except during observations Bees anaesthetised with CO2 and placed in 500ml plastic cages; 10 bees per cage, minimum 5 doses, 3 replicates per dose, fed treatment (fresh solution) 200µl 4 hrs/day for 10 days and *ad libitum* sucrose for remainder of day.	No toxic standard	Control (dosed with same level of solvent as in treatments)	Probit	Test not ring-tested
Belzunces (2006) Oral chronic toxicity tests (intermittent)	Young worker honey bees (*A. mellifera*)	Oral (intermittent exposure)	Mortal-ity	5 days (each day exposure is done as in acute toxicity	2h starvation 25° C ± 2°C Humidity 50-70% Dark	No toxic standard	3 control groups of 10 bees each Test valid if average mortality ≤10% in controls after 5 days 4 dose groups covering the LD50, 3 replicates	Effect-time curve, Mortality recorded every 24h up to 5 days. Calculation of	Study developed for the comparison of acute and repeated (intermittent)

Laboratory				testing)			with 10 bees each	the dose consumed	exposure

Table 5.3: Larval bees/acute or prolonged exposure

Test method	Life stage	Exposure route	Endpoint	Duration	Test Conditions	Toxic standard	Control No. of dose and replicates	Stat. evaluation	Comments
Aupinel (2007, 2009) Laboratory	Larvae A. mellifera	Oral exposure Test substance mixed into the diet of larvae	Mortality Emergence rate LD50, NOAEC, NOAEL	Up to 22 d	34°C Humidity 96% days 1-7 34°C Humidity 80% after day 7	Dimethoate	Control is included, including solvent control. Acceptable control mortality 15% (for LD50 and LC50) or 20% (for NOEAC and NOAEL), and successful hatch of adults in at least the control group 5 doses tested, 3 replicates	LD50 (24 and 48h, ug/larvae) calculated by probit analysis, 95% C.I. LC50 (ug/L solution). NOAEC and NOAEL calculated by chi-square test, level of significance 5%	Good control over exposure of larvae and environmental conditions potentially influencing test results, good measure of intrinsic toxicity, Larvae not reared in the natural environment

5.2.2 Analysis and identification of weaknesses

5.2.2.1 Sources of variation of the dose-effect relationship

The parameter LD50 has been traditionally used on the basis of its reduced variability (small Confidence Interval), considering that the dose-effect profile normally follows a sigmoid form. Furthermore, the methodologies for its estimation are aimed at ensuring its reproducibility. However, the validity of the LD50 as a toxicity measurement has already been questioned (Zbinden and Flury-Roversi, 1981). This parameter was created in 1927 for the biological standardization of dangerous drugs. However, even though the methodology is supposed to be standardised and results are thought to be reproducible, active substances have shown different acute mortality values among studies. The oral LD50 of Imidacloprid, for example, is observed between 3.7 and 40.9 ng/bee (48h; Schmuck et al., 2001), 5 ng/bee (48h; Suchail et al., 2000) or 40 and 60 ng/bee (48h and 72 h, respectively, Suchail et al., 2001), 49 and 102 ng/bee (48h; Nauen et al., 2001), and 490 ng/bee (48h; DEFRA, 2007). Fipronil showed oral LD50 from 4-6 ng/bee (48h, Aliouane *et al.,* 2008) to 123 ng/bee (48h; DEFRA, 2007). Contact LD50 may vary as well for each active substance.

Some of the sources of variation of the dose-effect relationship demonstrated in the scientific literature can be found in Appendix O.

5.2.2.2 Exposure through inhalation

Exposure through inhalation is not covered in laboratory conditions.

5.2.2.3 Intermittent exposure through oral route, contact or inhalation

When repeated exposure to a pesticide is considered (intermittent doses), the ratio acute/chronic oral toxicity shows a range from 0.05 (Emamectin) to > 1 000 (various active substances) (DEFRA, 2009; Illarionov, 1991; Belzunces, 2006). A possible explanation of these effects could be based on the ability of the active substance to induce the activity of detoxification enzymes. Namely, after the exposure to low doses administered frequently, some active substances could be detoxified while others could not. Repeated dosage might be more toxic for those active ingredients for which metabolization does not lead to activation rate. When active substances are metabolised leading to toxic metabolites, the difference between repeated and continuous exposure would not be as large (DEFRA, 2009). Therefore, the organism would be able to recover before an ulterior intoxication in the former case, which would not be the case of the latter. On the contrary, a unique exposure to higher doses may conduct to lethal effects because either the quantity of active substances is larger than the capacity of detoxification or the detoxification enzymes would not be induced.

The effect of repeated exposure to a pesticide depends not only on toxic kinetics, but also ergo kinetics and receptor binding (see Chapter 7). That is why it is not possible to foresee the toxicity behaviour following repeated exposure to toxic substances. The trials carried out during the comparative studies between the effects following a unique or a repeated dose (toxicity observed over 5 days after unique exposure) showed an increasing toxicity trend in substances like Clothianidine or Acetamiprid (Belzunces, 2006). Thiametoxam, on the contrary, did not show this trend.

Currently, intermittent exposure is not covered in risk assessment.

5.2.2.4 Prolonged effects inadequately covered

Investigation of chronic endpoints is currently incidental in the existing testing schemes.

Chronic toxicity can appear at doses below the LD50 (from 0.51 to 100 000). Furthermore, scientific studies have shown no clear correlation between acute and chronic toxicity. As a result, each active substance and PPP has toxicity behaviours (kinetics and dynamics) depending on its pattern of exposure (acute or chronic).

The potential contamination of bees' food sources (nectar and pollen) entails a continuous exposure of certain classes and castes of bees, namely those not leaving the hive. For this reason, additional testing of the chronic toxicity (following continuous exposure) would be recommendable as complementary to the acute toxicity.

Certain active substances have shown increased mortality over the observation period when calculating LD50 values. Suchail et al. (2001), showed prolonged action of Imidacloprid and two of its metabolites (olefin and 5-hydroxy-imidacloprd) for up to 96 hours, some of these substances showing an increasing tendency. The same is shown for fipronil sulfone, the oxidative metabolite of fipronil (Bocksch, 2003, 2004). The toxicity evolution beyond this observation period is not known as no longer observation period is prescribed. These effects might be the result of the long-term residual effectiveness of the mother compound or the bio-activation of toxic metabolites. A longer observation period could be envisaged after unique exposure as long as there is an evolution in toxicity, and the control mortality does not rise above unacceptable values.

5.2.2.5 Sub-lethal endpoints inadequately covered

Several authors have recommended the inclusion of tests evaluating sub-lethal effects for a complete analysis of the pesticide impact on bees (Thompson, 2003; Desneux et al., 2007, Pham-Delègue et al., 2002; Tasei et al., 2003). Research is required to enable measures of sub-lethal effect in the laboratory to be linked with effects at the colony scale. This is proposed as a limited battery of tests run in laboratory conditions.

Investigation of sub-lethal endpoints is currently incidental in the current testing schemes.

5.2.2.6 Toxic effects on larval life stage

The study of toxicity on larvae is not covered in conventional testing

5.2.2.7 Toxic effects on bumble bees and solitary bees

The toxicity on bees other than *Apis* bees is not covered in conventional testing.

5.2.3 Suggestions for improvements

Here, we deal sequentially with the limitations identified in section 5.2.2. Toxicity tests run in laboratory conditions should always be run in first tier in case the exposure of bees to the active ingredient cannot be excluded. The results of these tests will be used for the estimation of risk coefficients taking into account the possible exposure to the molecule under consideration.

5.2.3.1 Sources of variation of the dose-effect relationship

In an attempt to reduce variation of the dose-effect relationship as much as possible, it would be recommendable, during its estimation, to better fix the variables that may provoke variation. Specifically, it would be useful to have methodologies that are more prescriptive on the "bee collection process for testing". Factors that should be further considered for methodology improvement are the age of bees used in testing, temperature and hygrometry, syrup administration volume and toxic concentration, bee's subspecies, nutritional and health status, etc.

Furthermore, it is already requested that toxicity testing provides the EC_{10}, EC_{20}, EC_{50} and the slope (or an explanation if they cannot be estimated) together with the NOEC. Risk assessors could then estimate if the active ingredient requires special consideration depending on these data.

5.2.3.2 Exposure through inhalation

Inhalation is not an important exposure route to be considered in laboratory conditions (see Chapter 3), so no improvement needed.

5.2.3.3 Intermittent exposure through oral, contact or inhalation

Future research should be conducted in order to determine which of the two prolonged exposure patterns, intermittent or continuous, is the worst in terms of toxicity of these two cases.

5.2.3.4 Prolonged effects inadequately covered

Realistic exposures may be longer than the 96 h currently studied in conventional toxicity tests. As a result, chronic toxicity testing should be systematically evaluated in first tier of testing. When needed, risk assessors may consider lengthening the observation period for more than 96h in acute toxicity testing.

5.2.3.5 Sub-lethal endpoints inadequately covered

The scientific community recommends conducting the assessment of sub-lethal effects both at sub-lethal and lethal doses/concentrations (Desneux et al., 2007). The present section includes potential physiological and behavioural methodologies to evaluate the impact of pesticides on bees.

First, we propose a series of behavioural symptoms of intoxication that can be observed in bees during conventional laboratory toxicological tests. In future, it is recommended to complete the information on mortality gathered through acute and chronic toxicity tests by recording any intoxication signs, together with their duration, time of onset, severity and number of affected bees at each dosage level.

Scientific literature has already recorded several intoxication symptoms in laboratory tests as neurotoxicity symptoms, such as movement coordination problems, trembling, and tumbling, hypo-/hyper-responsiveness and hypo-/hyperactivity (Suchail et al., 2001), abnormal movements of legs or wings (Aliouane et al., 2008), etc. In semi-field/field conditions symptoms recorded by scientists and beekeepers may go from motionless bees on plant flowers or parts, limited cleaning of legs and horns, increased cleaning behaviour with middle or hind legs to heavy intoxication signs as bees hanging from leaves or flowers, paralyses and disordered wings or legs (Giffard and Mamet, 2009).

5.2.3.5.1 Behavioural sub-lethal endpoints: Proboscis Extension Reflex (PER) tests

We propose a behavioural test based on a different sub-lethal endpoint, the PER test (Table 5.4). The proboscis extension reflex method is a quantifiable and reliable method used for several purposes. It is based on the discovery of a Pavlovian reflex in the bee linked to the ability to memorise an odour (Kuwabara et al., 1957). It has been widely used in bee neurobiology research. The PER has allowed modelling the neural reflex (Menzel and Giurfa, 2001); to characterize the different levels of memory in the honey bee (Menzel, 2001); to assess bees' ability to discriminate between comb-waxes of differing ages (Fröhlich et al., 2000); to discriminate healthy brood from sick one (Gramacho and Spivak, 2003) or to measure the effect of sleep-deprivation on the memory capacity of the bee (Hussaini et al., 2009). Various laboratories have already used the PER test to assess toxicity at sub-lethal doses/concentrations (Pham-Delègue et al., 2002; Decourtye et al., 2005, Guez et al., 2001, Bernadou et al., 2009; CRA-API, 2010).

Arguably, the PER is a biologically relevant endpoint because the ability to recognise a variety of smells is vital to ensure, among others, foraging performance. Conditioned learning reflects food-location behaviour of free-flying foragers, enabling navigation and location of nectar sources over time (Pham-Delègue et al., 2002). Furthermore, studies developed within the project APEnet (CRA-API, 2010), tested the ability of bees to recognise several odours involved in colony cohesion, as queen pheromone components and linalool (Nassanov gland's component with functions of summoning foragers back to the colony and of aggregation). The exposure of bees to environmentally relevant doses of a.i. significantly hindered the capacity of identification of these odours. Moreover, odour discrimination plays a key role in the social immunity of the colony. Bees are able to identify a specific odour through the wax capping of sick brood, being able to extract and destroy infected

larvae. The so-called "hygienic bees" are genetic strains that have demonstrated to be more sensitive to the smell of contaminated brood (Gramacho and Spivak, 2003). Consequently, the PER is a crucial indicator for behavioural patterns that are vital to the survival of the hive.

The PER test is a quantifiable and reliable method to evaluate the impact of sub-lethal doses on bees' behaviour. The methodology is flexible enough to adapt to different exposure scenarios. Indeed, in order to optimise individual testing, survivors from the chronic toxicity tests could be used for testing, simulating the scenario in which exposure happens prior to the bioassay.

The fact that environmentally relevant concentrations of active substance lead to effects on the PER at individual level indicates that the colony survival might be at stake. Therefore, higher tier studies should be developed in which the toxicological endpoints should include observations on possible effects on the colony immune competence (bees' cleaning behaviour, undertaking, removal of sick larvae, etc.), queen performance or eventual requeening, number of foragers returning to the hive (homing flight) and foraging behaviour. Should higher tier studies not be carried out, pesticides affecting the PER should be considered highly toxic for bees.

The PER test is, therefore, a good tool to evaluate if pesticide exposure at environmentally relevant concentrations may entail a risk.

5.2.3.5.2 Physiological sub-lethal endpoints: molecular and histological markers

A very promising approach is the evaluation of the impact of pesticide exposure on different bee enzymatic and protein biomarkers or tissue development. In the same way as for humans, several enzymes can serve as biomarkers representing different metabolic ways or physiological functions. The exposure of bees to a pesticide or a pathogen may lead to a variation of certain molecules within the bees' tissues (Brunet et al., 2010). Hypopharyngeal glands tissue has been seen to be affected by the exposure to pesticides during the first stages of adult life (Smodis Skerl and Gregorc, 2010). However, such biochemical and histological modifications can still not be linked to dysfunction at colony level. The future development of these approaches as a diagnostic tool to predict the origin of a perturbation of the bees' vitality will definitely help improve the risk assessment.

5.2.3.6 Toxic effects on larval life stage

The test proposed by Aupinel (Table 4.3) is a "new" approach (quantitative, in laboratory conditions) when considering the traditional test methods in which the effects of pesticides on larvae were undertaken in semi-field or field conditions. Given the potential toxicological impact of pesticides during the larval period at colony level, it is recommendable to run laboratory larvae feeding tests in the first tier as larvae exposure to pesticides cannot be excluded.

The Working Group considered that there are a number of additional recommendations which should be made in relation to the study.

1. The Aupinel methodology (Aupinel et al., 2007 and Aupinel et al., 2009) was designed for use as either a single dose acute study with 48hr LD50 generated or a chronic 7-day feeding study leading to an LC50 for pre-pupal larvae. It is considered that the chronic dosing study is more relevant to the exposure of larvae in the hive than a single acute dose and this test design should be prioritised for consideration as the most appropriate test method for pesticide risk assessment. This would be specially the case whenever systemic products/active substances are considered or bees' food and water sources might result contaminated.

2. There are concerns over the use of dimethoate as the toxic reference for larval tests as the low solubility of the active ingredient in the royal jelly/sugar diet may result in poor uptake by the larvae. However, a positive control is used to confirm exposure and dimethoate might be considered a relevant active ingredient because of its effect on larvae.

A more relevant water-soluble active substance, preferably one known to be systemic, could also be used as a toxic reference.

3. The low solubility of active ingredients in royal jelly is an issue for a wider range of active ingredients and the acceptable solvent concentrations in the larval test should be addressed.

4. The 48hr study as currently proposed (Aupinel et al., 2009) has validity criteria of both less than 15% mortality in the control and successful hatch of adults in at least the control group. The Aupinel et al. (2009) paper shows that in 11 out of 31 cases less than 50% emerged adults were observed in the control. Given that the data will be used to generate LD50 and NOEC values, greater reliance should be placed on the negative control and toxic reference to confirm the validity of studies.

Differently from Oomen et al. (1992), the laboratory larval toxicity test allows a quantitative evaluation of the direct toxicity of pesticides to larvae under laboratory conditions, highly recommendable as a first tier test. It accounts for a number of other advantages: (1) run in controlled conditions and not in open-field, therefore provides more accurate measures of intrinsic toxicity; (2) tested product readily available to brood with no possibility to be stored in combs; (3) no possibility of dilution effect; (4) its acute version has undergone ring-testing and allows a quantitative estimation of the larvae toxicity, which is not the case for Oomen et al., 1992; (5) it is a much cheaper approach than running tests in semi-field/field conditions. OECD 75, is a test that has been validated for testing larvae toxicity under semi-field conditions and can be used in higher tier tests.

5.2.3.7 Toxic effects on bumble bees and solitary bees

The current RA scheme (EPPO PP 3/10) suggests making predictions for other bee species by extrapolation from the large body of data on honey bees. However, the extrapolation from data of honey bees to other bees may not be appropriate because the level of exposure to pesticides of non-*Apis* bees could be completely different from *Apis mellifera* (see Chapter 3) and because the level of sensitivity to pesticides may vary significantly between different bee taxa (Tasei, 2002; Devillers et al., 2003). In the SETAC Pellston scheme, non-*Apis* bees are included in the risk assessment, however the validation of the existing toxicity tests is necessary. A number of methods for testing the toxicity of pesticides to bumble bees have evolved over the last few years based on the established methods for honey bee toxicity testing. By far the majority of tests have been developed using *B. terrestris* which is the species used commercially for pollination and is therefore readily available (Table 5.5). For solitary bees, the tests available in literature have involved mainly few species: the cavity-nesting, spring flying *Osmia* spp. and summer flying *Megachile rotundata* and the ground-nesting *Nomia melanderi* (Table 5.6).

5.2.4 General conclusions and recommendations

1. When needed, risk assessors may consider it necessary to lengthen the observation period for more than 96h in acute toxicity testing.
2. An additional and more prolonged adult laboratory toxicity test should systematically be conducted (10 days exposure) as a first tier study.
3. Account should be taken during the risk assessment of all sub-lethal effects (intoxication signs) that are observed (e.g. feeding rate in oral dosing studies, effects on foraging in semi-field and field studies, etc.) and ultimately specific tests aimed at evaluating sub-lethal effects should be adopted in the first tier of testing.
4. An additional larval chronic (7 days) laboratory toxicity test should be undertaken in the first tier (e.g. Aupinel et al., 2007, 2009) for all substances to which larvae can be exposed and, in addition, for those substances with insecticidal properties, or which are shown to be cumulative in larvae. An Oomen et al. (1992) type study is required to integrate brood care

behaviour of the adult bees. IGRs should always be the target of further testing (Oomen *et al* (1992) or OECD 75).

5. Appropriate sub-lethal effect studies should be incorporated into future risk assessments. Potential first tier test methods to investigate sub-lethal effects have been identified as *Bombus* microcolonies (for effects on reproduction), PER (for neurotoxicity effects (memory capacity and learning)), and homing behaviour (for effects on foraging behaviour, including orientation, navigation, etc.). A priority for research is to determine the most appropriate sub-lethal endpoints for use in the first tier risk assessment, e.g. by correlating test endpoints with colony level effects such as PER with homing behaviour/foraging behaviour or observations on colony cohesion and functioning. Laboratory based studies on *Bombus* spp. micro-colonies show potential to be used as a screen for reproduction effects of products in the first tier. However, research is needed both to investigate whether such studies can be used, e.g. with an assessment factor, as a surrogate for honey bees and solitary bees and to integrate the results into a risk assessment scheme.
6. Development of molecular markers of toxic effects to enable prediction of potential sub-lethal effects such as immune system changes, potential interactions between products and effects in colony under real use conditions.
7. Laboratory toxicity tests on solitary bees should be included in the first tier. The available protocols are suitable to study the oral and contact toxicity in adults and larvae for several species of solitary bees. *Megachile rotundata* and several *Osmia* spp. show potential to be used as test species because their biology is well known and they are reared as crop pollinators.
8. More studies are necessary to compare the susceptibility of honey bees with other non-*Apis* species.

Table 5.4: Details of the test methodology to run Proboscis Extension Reflex tests in laboratory conditions

Test method	Life stage	Expo-sure route	Endpoint	Duration	Test Con-ditions	Toxic standard	Control No. of dose and replicates	Stat. evaluation	Comments
Decourtye et al. (2003, 2005)	Adult winter bees and new emerged honey bees (*A. mellifera*)	Oral exposure	NOAEC, NOAEL Learning behaviour	Newly emerged bees fed from 2 to 14–15 days old, the quantity of treated sugar solution provided daily is adjusted to the number of surviving bees. Bees starve for 4h prior to conditioning. Bees are selected for showing a proboscis extension reflex after stimulation of the antennae with a sucrose solution (300 g/L).	Darkness, 33 (+-2) °C, 40 (+-10)% RH Tests for acariosis, nosemosis, black disease, acute paralysis virus and spiroplasmosis are performed prior to testing.	Dimetho-ate	5 doses tested of each product 3 replicates Chemicals dissolved in sucrose solution (500g/L). Solvent (acetone) dissolved in the sucrose solutions at 10ml/L 60-80 bees/dose – 30-60 bees tested/ treatment Bees provided with sugar food (mixture of sugar and honey) and water *ad libitum* during the initial 2 days, and with pollen for the following 8 days.	Number of initial reflex responses and number of conditioned responses are compared between each concentration of the chemical and the control, by multiple two-by-two chi-squared tests with 1 df. When conditions of application of the chi-squared test are not fulfilled, Fisher's exact method is applied. 5% significance threshold divided by n, n being the number of comparisons with the same control data.	The methodology can include odours related with the cohesion of the colony as the queen pheromone, geraniool or linalool

Table 5.5: Details of the test methodology to run tests in laboratory conditions for bumble bees

Contact exposure	Oral exposure	Reference
1µl acetone dissolved pesticide formulation, ventral thorax 2nd-3rd pairs of legs, 10 bees per concentration, 5 concentrations, 24 hr mortality, link between bumble bee size and LD_{50}, linear regression		Van der Steen (1994)
	active ingredient dissolved in 50% sucrose, individually fed with calibrated pipettes, kept isolated, probit analysis mortality at 24, 48, 72 hrs, LD_{50} related to size of bee	Drescher and Geusen-Pfister (1991)
(sub-lethal test) Anaesthetised with CO_2, 1µl drop formulated pesticide in acetone on thorax,		Tasei et al. (1994)
30sec CO_2, 1µl drop formulated pesticide in acetone, controls acetone alone, applied to thorax, 8-10 bees per box 1dm^3 fed 35% sucrose. 20°C in dark, mortality checked daily	fed on sucrose containing pesticide, treatment 4 groups of 8 workers, mortality and uptake checked daily (corrected for evaporation)	Tasei et al. (1994)
	30µl formulated pesticide dissolved in 50% sucrose, offered to individuals in micropipettes. Then 72% sucrose *ad libitum*, kept in transparent cups 20°C, 55% RH, 4-6 doses, 30 bees per dose	Gretenkord and Drescher (1993)
As OECD 214	Formulated pesticide dissolved in 30% sucrose, fed to 10 bees of comparable body weight for 24 hrs	Schaefer et al. (1993)
mortality in control <=10%, mean weight of bees determined, anaesthetised for as short a time as possible, test substance dissolved in acetone, bees kept in dark at 25±2°C, 30 bees per concentration, 5 concentrations test substance, 2 replicates in time preceded by range finding test, 1µl test solution pipetted on ventral part of thorax between 2nd and 3rd pairs of legs, bees housed together by dose and fed sucrose solution *ad libitum*, mortality recorded 24, 48, 72 hrs,. Toxic reference 40% dimethoate or 25% parathion 3 concentrations and acetone control, LD_{50} µg/bee or µg/g bee.	30 bees per dose, mortality in control <=10%, bees individually caged for dosing, mean weight of bees determined, deprived of food 2-3hrs before dosing, not anaesthetised with CO_2, pesticide dissolved in sucrose, kept in dark at 25±2°C, 5 concentrations test substance, 2 replicates in time preceded by range finding, 10µl test solution fed so cannot be contaminated, 2 hr dosing period, after dosing bees housed together by dose, and fed sucrose *ad libitum*, mortality recorded, 24, 48 and 72 hrs. Toxic reference 40% dimethoate or 20% parathion, 3 concentrations and control, LD_{50} µg/bee or µg/g bee	Van der Steen et al. (1996)

Table 5.6: Details of the test methodology to run tests in laboratory conditions for bumble bees and solitary bees

References	Species/ Life stage	Expo-sure route	Endpoint	Duration	Test Conditions	Toxic standard	Control No. of dose and replicates	Stat. evaluation	Comments/Notes
Abbott et al. (2008)	*Megachile rotundata* and Osmia lignaria larvae	Oral (1)	Mortality rate, develop-ment duration, adult weight	Total development period	T = 29 °C during development	Not applied	Control group included Initial sample size is not indicated	GLMs were used to analyze difference in development time and weight. Survival until cocoon completion and adulthood were analyzed using chi-square tests.	(1) 1st method: 10 µL (1 µL for ALCB) of test solution/provision was applied; 2nd method: pollen provision replaced with a preblended pollen mixture containing the appropriate amount of a.i.
Charnetski (1988)	*Megachile rotundata* adult	Inhal-ation and indirect contact by walking on contam-inated surface (1)	Mortality rate	24 – 48 h	T = 28 °C (12:8 h photoperiod)	Not applied	Control group included 10 test bees were introduced into each of the tube sections replications.	Corrected mortality by Abbott's formula was analyzed with ANOVA using sex, treatment and hours as factors.	(1) A tube chamber constructed of clear plastic sheets forming a tube was separated by a screen partition into a top and a bottom section to test vapor and residue hazard, respectively.
Gradish et al. (2012)	*Megachile rotundata* adults and larvae	Topical (1) Oral(2)	Adults: Mortality rate Larvae: development and survival	Adults: 48 h; Larvae: development period	Adults: T = 25 °C Larvae: T = 30 °C (overwinter-ing at 6 °C)	Not applied	Control group included	Dose-response curve, median LC50 Larval mortality data were subjected to an analysis of variance using the	(1) 1 ml of test solution was applied using a Potter spray tower (PST). Bees were placed dorsal side up in the PST; (2) Pollen provisions of each leaf cell

								Mixed procedure in SAS. ANOVA was used to assess development duration and adult emergence time.	were injected with 1 µl of the appropriate test solution
Hodgson et al. (2011)	*Megachile rotundata* larvae (♀-♂)	Oral(1)	Egg and larva mortality rate	Up to 21 days	T = 27 °C; in darkness	Not applied	Two controls were used: an untreated water control and a blank treatment	The effect of treatment on mortality was assessed using a GLM.	(1) 1µl of different concentrations of the a.i. were applied to the provision mass.
Huntzinger et al. (2008)	*Megachile rotundata* adults (♀-♂)	Topical (1) Indirect contact (2) Oral(3)	Survival rate	Up to 20 days (topical and oral) – 40 days (indirect contact)	T = 29 °C; 12:12 h photoperiod	Not applied	Control group (water) included and no application treatment For each concentration, 3 to 14 replicates, each containing 10 bees	Survivorship analysis	(1) Different concentrations of the a.i. were applied to the bees thoraces. (2) Bees were allowed to walk on filter papers that had been treated with different concentrations of the a.i. (3) Bees were fed sugar solutions treated with different concentrations of a.i.
Johansen et al. (1983), see also Johansen (1972)	*Megachile rotundata* and *Nomia melanderi* adult (1-day-old	Indirect contact by walking on contami	RT25: residual time required to obtain a bee mortality of 25% after a	Mortality level: 24-48-72 h;	29.5-30°C; 60% RH.	Not applied	Control group included 4 replications for each	The bee mortality rate was recorded at 24, 48 and 72 h for various ages of residues to establish the	(1) Foliage was sampled from alfalfa plots previously treated with the test insecticides and

	adult) (♀)	nated surface (1)	test exposure to pesticide application				treatment and time interval with 20-40 bees	RT25.	placed in a cage with bees
Johansen et al. (1984)	*Megachile rotundata* and *Nomia melanderi* adults (♂-♀)	Oral (1)	Mortality Acute LD50 and chronic LC50	Acute toxicity test: 24-48-72 h; Chronic feeding test: up to 21 days	29.5°C; 60% RH.	Not specified	Control group included 20 bees were individually treated per dosage level	LD50 and LC50 values were computed by means of Probit analysis	(1) Bees were individually fed amount of test solutions placed into tubes inserted in caps of glass vials. 10 bees were grouped after dosing.
Konrad et al. (2008)	*Osmia bicornis* (=*O. rufa*) larvae	Oral (1)	Larval develop-ment, mortality, body weight and adult longevity	From egg to adult	T = 20 ± 1°C; 75 ± 5% RH, no light	Not applied	Control group included		(1) 50 µL of test solution/g of provision was applied into a longitudinal fissure in the provision mass
Ladurner et al. (2003, 2005)	*Osmia lignaria* and *Megachile rotundata* adults (♀) (~ 24 h after emergence)	Oral (1) and contact (2) expo-sure	Acute toxicity: Mortality Acute LD50 Delayed toxicity: Survival rate	Acute toxicity: 24-48-72 h; Delayed toxicity: up to 7 days	T = 22 °C (O. lignaria) – 25 °C (M. rotundata); RH = 60-80% Photoperiod (L:D = 12:12 h)	Dimeth-oate was used as standard toxic	Control group included; 5 doses tested of each product 3 replicates of 10 bees/doses	Acute toxicity: Dose-response curve, median LD50, 95% C.I.; Delayed toxicity: Survival analysis	(1) Bees were individually fed 10 µL of test solution using the flower method. For delayed activity fed on fresh sucrose solution. (2) 1 µL of test solution/bee was applied to the dorsal surface of the thorax.
Mayer et al. (1998); Mayer	*Megachile rotundata*	Topical (1)	Mortality rate	24 h	T = 26 to 29°C; 50%	Not applied	Control group included	Topical: Dose-response curve,	(1) 2µl of different concentrations of

and Lunden (1999)	and *Nomia melanderi* adults (♀)	Residual contact (2)			RH		20 females/ species/treat-ment	median LD50 Residual test: 24 h mortality (%)	the a.i. were applied on to the thorax; (2) Foliage was sampled from alfalfa plots previously treated with the test insecticides and placed in a cage with bees
Peach et al. (1995)	Megachile rotundata larvae	Oral (1)	Size (body weight) of emerged adults	Development period	T = 30 °C and 50% RH (overwinter-ed at 4 °C).	Not applied	Control group included 40 bees/treat-ment. Experiment repeated 3 times.	ANOVA was used to compare body weight	(1) 1-2 mg of test solution was applied in the provision mass
Scott-Dupree et al. (2009)	*Bombus impatiens, Megachile rotundata* and *Osmia lignaria* adults (♀-♂)	Direct contact (1)	Mortality acute contact LC50	48 h	T = 25 ± 1°C in the dark	Not applied	Control group included For each concentration, four to six replicates, each containing nine to 11 bees, were tested	Dose-response curve, median LC50	(1) 5 ml of test solution was applied using a Potter spray tower (PST). Bees were placed ventral side up in the PST.
Tasei (1977)	*Megachile rotundata* (= *pacifica*) adult (<48 h from	Indirect contact by walking on	Mortality rate	24 h	27 ± 1°C under constant light. No food is	Not applied	Control group included 4 replications	The mortality rate was corrected by Abbott's formula and compared among treatments.	(1) Filter papers soaked in test solution, dried and placed on the bottom of screened

	emergence) (♂)	contaminated surface (1)			provided		for each treatment with 12-15 bees		boxes
Tasei et al. (1988)	*Megachile rotundata* adults (♀-♂) (< 48 h after emergence)	Contact (1)	Mortality Acute contact LD50 and LD1 Longevity at sub-lethal doses (<LD1)	Acute toxicity: 48 h Mortality rate: Up to 7 days from application	25° C ± 2°C	Not applied	Control group included 7 doses and 6-5 replications with 15 bees each	Dose-response curve, median LD50 and LD1.	(1) 1 µL of test solution/bee was applied to the dorsal surface of the thorax.
Tasei et al. (1988)	*Megachile rotundata* larvae (♂)	Oral (1)	Larval mortality rate Larval development duration and diapause incidence	Larval development period	30°C	Not applied	Control group included 4 doses with 15 bees each	Larval mortality rate, development duration and diapause rate was compared among doses.	(1) 1 µL of test solution/provision was applied
Tesoriero et al. (2003)	*Osmia cornuta* larvae	Oral (1)	Mortality rate during development	From egg to last larval stage	T = 23 ± 1°C; 70 ± 10% RH	Not applied	Control group included (untreated control) plus non-manipulated control About 30 bees per treatment	Percentage of larval survival was analyzed using chi-square tests.	1) 1 µL of test solution at field dose/provision was applied;

5.3 Semi-field tests

5.3.1 Summary

Semi-field testing (cage, tunnel or tent tests) have been regarded as higher tier studies that may be triggered in the current risk assessment scheme used in the EU as a result of the standard Tier 1 risk assessment i.e. contact or oral hazard quotients >50. Semi-field studies are often also called tunnel tests.

Three test designs have either been traditionally used or validated for testing the impact of pesticides in the semi-field, the EPPO 170 (4) semi-field test, the OECD 75 brood guidance document and the Oomen et al. (1992) brood feeding study.

The EPPO 170 also gives guidance on conducting semi-field tests. Such studies are often used to investigate the acute effects on foragers and hive bees following spray applications, but the semi-field testing study design can also be modified for specific assessments of honey bees, e.g. repellency and other behavioural effects, timing of application, effects of aged residues, evaluation of the hazard of applying plant protection products to honey bees foraging the honeydew secreted by aphids or specific testing of brood effects.

The OECD Guidance Document 75 describes the Honey Bee Brood Test conducted under semi-field conditions with a quantitative assessment of adverse effects of plant protection products on the development of the honey bee brood.

The Oomen test is designed for investigation of effects following oral exposure of adult bees and especially of oral exposure of bee brood. The test is not a strict semi-field test as usually conducted in field conditions, thus larger colonies can be used but has also been conducted in semi-field conditions, with some adaptations e.g. colony size. In contrast to semi-field studies following EPPO 170 and OECD 75 with bee attractive crops, where bees are allowed to forage nectar and pollen, in the Oomen test the test item is directly fed with sugar solution inside the colony thus it can be regarded as an intermediate test, as in between extended lab and semi-field/field test as the doses can be controlled and defined concentrations are fed. In general, it is impossible to evaluate all the possible exposure routes of SST in a single semi-field trial, e.g. dusts, guttation and long-term exposure.

5.3.2 Semi-field test designs

Test method	Life stage	Exposure route	Endpoint	Duration	Test Conditions	Toxic standard	Control No. of dose and replicates	Stat. evaluation	Comments
EPPO 1/170 (4) Side effects on bees Semi-field tests	Colonies Approx. 3000-5000 bees	e.g. application during bee flight on bee attractive crops. Oral and contact exposure	Flight and / or foraging activity; general behavior of bees on the crop and around hives, mortality of bees at hive and on linen sheets in the crop; colony status / development (adults and brood)	Pre- application. 2-3 days Exposure period: 7 days in tunnel, 19 days observation outside tunnel Exposure period: flight, behavior and mortality at several intervals, preferably daily but at least 0, 1, 2, 3, 5 and 7 days after application. In-hive assessments up to 28 days on an approximately weekly basis	Crop size >40 m² Treatments should be applied e.g. when the test crop is in full flower during the daytime when bees are demonstrated to be actively foraging on the test crop but, depending on study aim, modification of set-up possible (e.g. treatment after daily bee flight)	Depending on study aim; Toxic standard with high hazard to bees (e.g. Dimethoate for acute toxicity; Fenoxycarb for IGR´s	Product(s) to be tested, toxic reference and an untreated control. Normally, the minimum number of replicates should be three in order to enable statistical analysis, but a lower number may be appropriate in some cases, for example with crops that need a large area (e.g. orchard trees)	Statistical analysis should normally be performed using appropriate methods, which should be indicated. If statistical analysis is not used, this should be justified. When interpreting the results, it needs to be recognized that there are endpoints which are intrinsically suitable for statistical evaluation (e.g. mortality data), whereas others may be not (e.g.behavioral endpoints).	Also methodology for field trials described (see field chapter)

Test method	Life stage	Exposure route	Endpoint	Duration	Test Conditions	Toxic standard	Control No. of dose and replicates	Stat. evaluation	Comments
OECD75	Small colonies, approx. 6000 adults, 3000 brood cells (750cm²)	e.g. application during bee flight on bee attractive crops. Oral and contact exposure	At least 100 eggs marked, development assessed flight and / or foraging activity; general behavior of bees on the crop and around hives, mortality of bees at hive and on linen sheets in the crop; colony status / development (adults and brood)	Cells assessed 5, 10, 16, 22 days Colony assessment 5, 10, 16, 22, 28 days Set-up at least 3 days pre-application. 7 days exposure period in tunnel, 19 days observation outside tunnel. Total duration at least 28 days	(Crop size >40 m² Treatments should be applied e.g. when the test crop is in full flower during the daytime when bees are demonstrated to be actively foraging on the test crop	Fenoxycarb	Normally, the minimum number of replicates should be three in order to enable statistical analysis. No control criteria stated.	No specific statistical analysis identified, but in line with OECD (2006).	Can be readily adapted (and is also used) for field studies Recent evaluation of potential method-ological improve-ments in Defra project PS2367; an evaluation by German bee group will be published in proceedings of ICPBR meeting 2011

Test method	Life stage	Exposure route	Endpoint	Duration	Test Conditions	Toxic standard	Control No. of dose and replicates	Stat. evaluation	Comments
Oomen et al (1992)	Colonies 10,000-15,000 bees, 18,000-25,000 brood cells	1 litre sucrose containing active ingredient directly fed to hive Colonies free flying, avoid high nectar flow	100 eggs, 100 young larvae, 100 old larvae assessed on marked acetate sheet and development assessed pupae extracted just prior to emergence to assess morphological abnormalities Adult and brood mortality	21 days after feeding Cells assessed weekly for 3 weeks, usually 7, 17, 19, 21 days, Daily mortality assessments	Free flying colonies. In principle, with adaptions (e.g. additional pollen supply also possible in tunnels) Single feeding of sucrose containing formulated product	Fenoxycarb or diflubenzuron	Concentration of active substance in sucrose solution at field dose Normally, the minimum number of replicates should be three in order to enable statistical analysis, Control mortality <15%	No specific statistical analysis identified	Recent evaluation in Defra project PS2367 to adapt to use with non-IGR compound and link to field residues

5.3.3 Analysis and identification of potential weaknesses

5.3.3.1 EPPO 170

- Limited size of crop area
- Methods to be used for statistical evaluation are not specified.
- Only limited potential to extrapolate from quantitative effects on bee brood to the findings on larger colony sizes used in field studies.
- Necessary methodological adjustments of the method e.g. for assessment of guttation and dusts which are currently not described in the guidelines.
- Some nectar and pollen stores need to remain in the hive to prevent starvation; this could reduce or delay potential exposure to nectar and pollen of the test item.
- No direct assessment of the exposure of individual bees possible.
- Standard studies are conducted over a relatively short timescale (one brood cycle).
- Details on further investigation of bee diseases and colony health status not sufficiently described.

5.3.3.2 OECD 75

- Limited size of crop area.
- Methods to be used for statistical evaluation are not specified.
- Acceptable control and toxic reference mortality criteria not given in the guidance document.
- Only limited potential to extrapolate from quantitative effects on bee brood to the findings on larger colony sizes used in field studies.
- Necessary methodological adjustments of the method e.g. for assessment of guttation and dusts which are currently not described in the guidance document.
- OECD Guidance document is directed primarily at the development of eggs (although it can be extended).
- Some nectar and pollen stores need to remain in the hive to prevent starvation; this could reduce or delay potential exposure to nectar and pollen of the test item.
- Standard studies are conducted over a relatively short timescale (one brood cycle).
- Acetate sheets used for documentation of larval development.
- Details on further investigation of bee diseases and colony health status not sufficiently described.
- The brood termination rate (= mortality of bee brood in selected cells on combs) may be subject to a certain degree of variation.

5.3.3.3 Oomen et al. (1992)

- Published method, no guidance available.
- Methodology has not been formally ring-tested and has not been validated.
- Method was primarily described for field tests with colony size of 10 000-15 000 bees. Lack of description of necessary adaptations for use in semi-field conditions, (e.g. smaller colonies).
- Methods to be used for statistical evaluation are not specified.
- The protocol is less detailed compared to EPPO 170 and OECD 75.
- Concentrations in pollen returned to the hive are not taken into account.
- Need for identifying suitable dosing levels in the Oomen et al (1992) testing.
- Acceptable control and toxic reference mortality criteria not given (but criteria when tests need to be repeated).
- Some nectar and pollen stores need to remain in the hive to prevent starvation; this could reduce or delay potential exposure to nectar and pollen of the test item
- Acetate sheets used for documentation of larval development.
- Details on further investigation of bee diseases and colony health status not sufficiently described.

5.3.4 Suggestions for improvements

5.3.4.1 EPPO 170

- Specific guidance on assessment of different categories of behavioral effects should be provided.

- It is recommended to develop methodologies adapted to pesticides applied as SSST.

- Consideration should be given to extending studies where significant exposure is likely to occur over a period longer than a single brood cycle, e.g. systemic or highly persistent residues. Acute effects are likely to occur on the short time scale evaluated by the study but the effects of longer term low-level exposure which may weaken colonies over several brood cycles, e.g. the longevity of emerging adults, changes in normal behaviour in progression from nurse bees to foragers or effects on the queen, e.g. reduced queen fecundity and egg laying rate, which may result in reduced over-wintering survival, may be less readily evaluated.

- Acute effects are likely to occur on the short time scale evaluated by the study but the effects of longer term low-level exposure which may weaken colonies over several brood cycles, e.g. the longevity of emerging adults, changes in normal behaviour in progression from nurse bees to foragers or effects on the queen, e.g. egg laying rate, which may result

in reduced over-wintering survival may be less readily evaluated. These effects are likely to be best addressed by purpose-designed studies, e.g. by addition to existing guidelines.

- Some methodological adjustments have recently been tested in research activities and proven to demonstrate potential risks e.g. for dust drift and guttation. It is encouraged that these recommendations be published and considered for developing further guidance.

- It should be mentioned in the document that underbasket mortality should also be assessed at regular intervals before and after exposure of the test item.

- Modifications of the forage area and size of colonies according to the study aim e.g. for screening seem reasonable but it is recommended to point out that standard studies should usually be conducted with larger colonies and sufficient effective forage area > 60 m², preferably > 80 m².

- It is recommended that further guidance on statistical evaluation should be provided, ensuring appropriate methods are used.

- The minimum number of replicates is recommended to be at least 4 in order to facilitate interpretation.

- It is recommended to add the current state of knowledge on a number of issues to the existing document, e.g. more detailed description and categorization of behavioural assessments, investigation of sub-lethal and delayed effects for purpose-designed studies. Depending on study aim, further endpoints should be described for potential investigations of special effects e.g. lifespan of hatching bees could be investigated.

5.3.4.2 OECD 75

- Control and toxic reference mortality criteria should be defined
- It is recommended that young and old larvae, as well as eggs, should be included in assessments of effects on brood in all studies.
- It needs to be assured that the assessment schedule is done with adequate frequency so that it is not possible that the natural cleaning of dead larvae or pupae from cells by bees masks effects. Therefore it is recommended that the OECD Guidance document is extended to assess adverse effects on all 3 stages of brood by marking 100 eggs, 100 young larvae and 100 old larvae per colony. There are significant advantages to interpretation if the effects of pesticides on eggs, young larvae and old larvae are assessed, so this should be included in assessments of effects on brood in all studies.
- Underbasket mortality should be assessed at regular intervals before and after exposure.

- Modifications of the forage area and size of colonies according to the study aim e.g. for screening seem reasonable but it is recommended to point out that standard studies should usually be conducted with larger colonies and sufficient effective forage area > 60 m², preferably > 80 m².
- Assess contents of all cells including deformities in pupae; weight of pupae should also be assessed to determine any adverse effects on development, e.g. delayed development.
- Use digital imaging instead of acetate sheets. Methods should be updated to this more accurate approach.
- Consideration should be given to extending studies where significant exposure is likely to occur over a period longer than a single brood cycle, e.g. systemic or highly persistent residues. Acute effects are likely to occur on the short time scale evaluated by the study but the effects of longer term low-level exposure which may weaken colonies over several brood cycles, e.g. the longevity of emerging adults, changes in normal behaviour in progression from nurse bees to foragers or effects on the queen, e.g. reduced queen fecundity and egg laying rate, which may result in reduced over-wintering survival, may be less readily evaluated.
- Levels of *Varroa* should be documented at regular intervals and samples of virus analyses need to be inspected, as examples for some further specification information which should be provided and sampling harmonized.
- Some methodological adjustments have recently been tested in research activities and proven to demonstrate potential risks e.g. for dust drift and guttation. It is encouraged that these recommendations be published and considered for developing further guidance.
- Specific statistical analysis for bee trials in semi-field and field conditions is still under development. In general it is recommended to follow the OECD guidelines (OECD, 2006). It would be highly desirable that further guidance on the appropriateness of methods and statistical evaluation for bee trials be elaborated and added to the guidance.
- To reduce variation, certain improvement factors were identified and should be included, e.g. to use bigger colonies with 3 to 4 brood combs, containing a high amount of capped brood, to start, if possible, studies early in the season, to avoid major modifications of the colonies shortly before application, to observe 200 to 400 cells and to water the crop if dry conditions reduce nectar flow.
- The minimum number of replicates is recommended be at least 4 in order to facilitate interpretation.

5.3.4.3 Oomen et al. (1992)

- The Oomen test has been routinely used for a number of years for generation of regulatory data and there is a significant database on control and toxic reference data and defined

control validity data. Thus, ring-testing and validation of the method should be conducted.

- It is recommended to encourage a comparison of available effect data of Oomen trials and OECD 75 studies to investigate if a worst case exposure of the larvae is achieved and to ensure effects obtained are comparable to effects of spray applications on flowering crops.

- Residue analyses of larvae should be conducted on a set of Oomen and OECD 75 or EPPO 170 studies to investigate if the exposure via fed sugar solution resembles exposure levels following applications of flowering crops.

- Refine description of the methodology.

- There are significant advantages to interpretation if the effects of pesticides on eggs, young larvae and old larvae are assessed. The approach makes it possible to investigate effects which may occur at different stages, e.g. whether young larvae are more sensitive than older larvae. It needs to be ascertained that the assessment schedule is carried out frequently so that the results are not masked by the natural instinct of the bees to clean the dead larvae or pupae from cells.

- Depending on the study aim, further endpoints should be described for potential investigations of special effects e.g. lifespan of hatching bees could be investigated.

- It is recommended that the Oomen method is extended to assess adverse effects on all 3 stages of brood. There are significant advantages to interpretation if the effects of pesticides on eggs, young larvae and old larvae are assessed, so this should be included in assessments of effects on brood in all studies. It needs to be assured that the assessment schedule is done with adequate frequency so it is not possible the natural cleaning of dead larvae or pupae from cells by bees can be masking observations.

- Use digital imaging instead of acetate sheets: methods should be updated to this more modern approach.

- Extending studies where significant exposure is likely to occur over a period longer than a single brood cycle, e.g. systemic or highly persistent residues.

- The longevity of emerging adults, changes in normal behaviour in progression from nurse bees to foragers or effects on the queen, e.g. egg laying rate, which may result in reduced over-wintering survival may be less readily evaluated. These effects are likely to be best addressed by purpose-designed studies, e.g. by addition to existing guidelines.

- For harmonisation and better comparability of Oomen studies to OECD 75 studies, for

example, it would be desirable if both the Oomen method and OECD 75 were harmonized for one toxic standard and application rate. It is proposed to use Fenoxycarb as a toxic standard because it has been widely used and due to the range of effects that can be observed.

- The Oomen et al. (1992) method allows a more flexible design to be developed in that a range of doses can be used and related to residues detected following field applications in a range of crops. Therefore a possible approach would be to assess the residues that are returned to the hive, in pollen loads and the honeysac of returning foragers, for representative crops and application rates from semi-field or field trials with target crop(s) and base dose rates in Oomen tests around these residue values to develop a dose response and NOEC or ECx. Such an approach would allow extrapolation to a range of crops and usage scenarios without additional brood studies.

- It is recommended to encourage a comparison of available effect data of Oomen trials and OECD 75 studies to investigate if a worst case exposure of the larvae is achieved and to check that the effects obtained are comparable to effects of spray applications on flowering crops.

- Results should be analysed with appropriate statistical methods. It would be highly desirable that further guidance on appropriateness of methods and the statistical evaluation for bee trials is elaborated and given for semi-field and field trials.

5.3.5 When to use which test (to address which questions)

5.3.5.1 General assessment of effects on honey bee larvae

- Either by the *in-vitro* larvae test (Aupinel test, see 5.2) or by the Oomen Test (Oomen is considered as intermediate between lower (Lab) and higher tier tests (Semi-field and Field).

- For assessing effects on honey bee larvae, the Aupinel or Oomen- Test can be skipped and higher tier tests on larval development (OECD 75) can be conducted in semi-field (and possibly field testing).

- For IGR´s, an Oomen Test or OECD 75 is always required and cannot be skipped. If concern is raised from findings from the Oomen test, further semi-field and field testing (OECD 75) is required.

5.3.5.2 For assessment of effects on adults and/or honey bee larvae after higher tier tests have been triggered:

- If concern for larvae only was raised from results from Aupinel or Oomen, semi-field (and

possibly field testing) (OECD 75) is required.

- When laboratory studies trigger higher tier tests due to concerns on adult bees only, semi-field (and possibly field testing) (EPPO 170) with brood assessments is required.

- If concerns for both adults and larvae are raised, a combination of EPPO 170 and OECD 75 (and possibly field testing) is required. (Daily mortality assessments for 28 days and special brood assessments as described in OECD 75.)

5.3.6 General conclusions and recommendations for all semi-field tests:

Semi-field testing (cage, tunnel or tent tests) appears to be a useful option before full field testing. The exposure is worst-case and more intensive than in the field (bees/colonies confined and forced to forage on the treated crop) and potential mortality is easy to assess. For accurate quantification of exposure, semi-field studies may provide suitable and reproducible information on residue levels both for sprayed products and also for residues following seed treatments or soil applications with systemic compounds. Semi-field studies aim at assessing the level of effects that may be expected on bees exposed to the product under realistic use conditions when the target crop has been treated. Semi-field testing should be designed to address and reproduce the route(s) of exposure of bees and the maximum level of exposure expected by these routes, as a result of a spray or of the presence of residues in flowers (nectar/pollen).

For sprayed products, semi-field tests may be used for demonstration of acceptable or unacceptable effects in a semi-field test using a worst-case flowering crop, in some cases also standard crops (i.e., wheat) which have been made artificially attractive through a sugar solution and treated at the maximum application rate. For assessing the effects of crops which might have low numbers of flowers per m² (e.g. zucchini) a worst-case flowering crop like *Phacelia tanacetifolia* is recommended to be used for testing potential risks assuming worst-case exposure.

All test systems seem to have some advantages but also some disadvantages. It seems no test system can be used to answer all questions in a single test.

In semi-field trials, due to refined foraging possibility on the treated crop, bees are forced to forage on the treated crop which can be assumed as a worst case scenario. The aim of a semi-field study could be to test the potential effects of a pesticide under more realistic conditions compared to the laboratory environment. As an advantage compared with the laboratory studies, semi-field studies present a more realistic environment; more information on behavioural interactions between the bees and their environment is obtained. Compared to field studies, semi-field studies are easier to control, have a better reproducibility and the interpretation is easier than for field studies and it is feasible to have a higher numbers of replicates which facilitates statistical evaluations. As the environment is controlled, some stressors may be especially well assessed in a semi-field study, e.g. contamination of water supply.

Nevertheless, semi-field test methodologies have some limitations e.g. due to the limited possible flight range and the limited possible study length in confined conditions, as it is hardly possible to keep colonies prospering in an enclosed structure for long periods. Confined conditions may also result in possible stress on bees which may result in a natural reduction of brood activity when bees are kept inside tunnels for longer periods. Due to the small size of the colony it is not possible to assess pollen and nectar storage and hive weight development; therefore, it is difficult to assess potential effects on honey production (i.e. a potential protection goal) when adverse effects are

observed on other parameters. As smaller sizes of colonies are used in semi-field studies, the interpretation of impacts on full size colonies will be difficult and the possibility to translate adverse effects on a small colony to a full size colony seems limited. Due to confined conditions, the small size of the colony and the high turnover rate of nectar and pollen in the tunnels, it is not possible to assess hive weight development and direct potential effects on honey production.

For all test systems in the semi-field, it is necessary that all categories of bees are thoroughly exposed and proof of exposure and consumption of the test item needs to be provided for all categories of bees. As with any other test, it is important to match the size of the colony to the area of forage available and the purpose of the study and minimise the levels of stores within the colonies. The use of small colonies is required in the semi-field methodology due to limited forage area. The use of small colonies is principally suitable also to detect effects on adult bees and bee brood, but has some limitations e.g. for assessing quantitative effects on brood nest development of large colonies, which are required in field conditions. Depending on the study aim, further endpoints e.g. specific behaviour, lifespan of hatching bees can be addressed in all studies for investigation of special effects. Consideration should be given to extending studies where significant exposure is likely to occur over a period longer than a single brood cycle, e.g. systemic or highly persistent residues. Adaptations of the tests for SSST should be elaborated, including a refined exposure assessment demonstrating proof of exposure. Young and old larvae, as well as eggs, should be included in assessments of effects on brood in all studies.

Improvements on a number of specific issues not yet included in the currently described methodology and procedures have been identified by this EFSA Working Group and also by other working groups. They should be considered and added to existing documents, e.g. more detailed description and categorization of behaviour assessments, investigation of sub-lethal and delayed effects and statistical analyses. Furthermore, experience on adaption of studies, e.g. for guttation and dust studies, should be included. Furthermore, there are numerous articles available in scientific literature describing the possible methodologies to test different sub-lethal effects. Thus further development of guidance on methodologies addressing specific issues is needed. In order to harmonise procedures, future ring-testing of the methods should be envisaged.

5.3.7 Semi-field test for social non-*Apis* (bumble bees) and solitary bees

5.3.7.1 *Bombus* spp.

By far the majority of higher-tier studies in bumble bees have been conducted in glasshouses due to the widespread use of bumble bees for pollination. There are no formalised guidelines but a number of methods using glasshouse conditions have been published, e.g. Gretenkord and Drescher (1996), Thompson and Barrett (2001), Tasei et al. (2001) and these have been reviewed by van der Steen (2001). Small bumble bee colonies are placed in glasshouses containing flowering plants treated with pesticide. Due attention needs to be paid to the age of the colony and the duration of the study due to the lifecycle of colonies, e.g. *Bombus terrestris* colonies are active for 5-6 weeks. Assessments of effects include adult and larval mortality (not all larvae are ejected from the hive), number of workers and brood and foraging activity. Assessment on individual larvae in a similar approach to the honey bee brood test is unlikely to be routinely applied due to the construction of the bumble bee nest. Only limited further work is required, e.g. guidance confirmation of toxic reference and control criteria to enable guidelines for semi-field studies to be agreed.

5.3.7.2 Solitary bees

There are no standardized guidelines but a number of methods have been published to test pesticides on solitary bees in cage, tunnel or glasshouse conditions (e.g. Tasei and Dinet, 1981; Tasei et al., 1988; Peach et al., 1995; Ladurner et al., 2008; Hodgson et al., 2011). In these studies, from 10 to 40

marked females (plus a number of males) were released in each cage containing flowering plants treated with pesticide. *Melilotus alba* or *Phacelia tanacetifolia* were often used as a test plant in semi-field studies because of its abundant flowering. Control cages with plants treated only with water were always included. Assessments of effects include survival and fecundity of nesting females, cell production rate, larval mortality and foraging activity (foraging time and in-nest times of nesting females). The relative repellent effect of two pesticides on nesting leafcutting bee females was assessed counting the number of bees on flowers in treated and control plants (Tasei and Dinet, 1981). The available protocols are limited to only two species, *Megachile rotundata* and *Osmia lignaria*, but they are suitable for standardization of the test guidelines in several related species. Only limited further work is required, e.g. guidance confirmation of toxic reference and control criteria to enable guidelines for semi-field studies to be agreed.

5.4 Field tests

5.4.1 Overview table with the tests

The only official guideline is the document EPPO 1/170 (4), Side effects on bees, Field tests.

Test method	Life stage	Exposure route	Endpoint	Duration	Test Conditions	Toxic standard	Control No. of dose and replicates	Stat. evaluation
EPPO 1/170 (4) Side effects on bees Field tests	Colonies at least 10 000 individuals	Mainly foragers	Flight and/or foraging activity; general behaviour of bees on the crop and around hives; mortality of bees; colony status / development	- Field observations should be conducted at several intervals, preferably daily but at least 0, 1, 2, 3, 5 and 7 days after application. - In-hive assessments should be conducted up to 28 days on an approximately weekly basis	The colonies should be placed in or on the edge of the flowering crop. The colonies should be in position approximately 2–3 days before the trial. Treatments should be applied when the test crop is in full flower during the daytime when bees are demonstrated to be actively foraging on the test crop	A toxic standard is usually not suitable for field trials.	Product(s) to be tested and an untreated control. Although very desirable, replication is often not feasible because of the requirements for separation	It has to be recognized that statistical analysis may not be feasible

5.4.2 Analysis and identification of potential weaknesses of field tests (EPPO 170)

A precise analysis of this guideline, based on scientific knowledge, showed that it is mainly aimed at evaluating the side-effects of spray products on honey bees, and it requires further adaptation and guidance to evaluate the side-effects of systemic compounds used as seed- and soil-treatments (SSST). This difficulty is mainly related to the fact that the time course of exposure differs for these two kinds of preparations: a short period for the sprayed non-systemic products (as little as a few hours up to several days, depending on the persistence of the active ingredient) versus a long period for the SSST (up to several weeks depending on the flowering period of the crop). This difference leads to important consequences on the quantity of contaminated food (nectar and pollen) brought back to the colonies. To address these issues several more detailed guidelines should be developed, each adapted to a specific mode of application of the pesticides, spray or SSST, systemicity, and to the real exposure routes of the honey bees to the specific exposure routes (e.g.dust during sowing operation, guttation).

The parameters within the field tests (see below) have been developed over the last 30 years and the original basis for these have not been not well documented scientifically.

For spray application: although there is a large database of studies providing assurance that the study designs are applicable, there are some further changes that would be beneficial.

For SSST: there is a far smaller database of field studies and so a greater definition of parameters, which more closely reflect the time course and routes of exposure in the different categories of bees (foragers and the in-nest bees including adults and immatures of colonies), is recommended.

For the analysis and identification of potential weaknesses, the comments concerning the spray products ("Sprayed products") will generally be separate from that of the systemic compounds used as seed- and soil-treatments ("SSST"). The systemic compounds applied as sprays (e.g. pre-flowering applications in orchards) must be evaluated using both the guidelines of the spray and of the SSST.

5.4.2.1 Choice of the colonies

Colony size

A population of 10 000 individuals is identified as the minimum size and is not representative of a normal colony during the spring and summer seasons, which is between 20 000 (spring) and 60 000 or more (June - July) individuals (Jeffree, 1955; Sakagami and Fukuda, 1968; Michener, 1974). A colony of 10 000 individuals corresponds to the beginning of its development at the end of the over-wintering period in Europe when it starts rapid expansion in the early spring. Field applications may occur not only in the early spring but also later in spring and during summer when larger sized colonies are more representative. However, during these periods beekeepers may divide their larger colonies in small ones in order to increase the number of their colonies.

- A general tendency in scientific literature is to consider that well populated colonies have more foragers than small colonies (Philips, 1930; Dirks, 1946; Reed, 1960; Lecomte, 1968), but Free (1960) has a contrary opinion. Farrar (1973) and Harbo (1986) showed that large populations are more efficient honey producers and yield more honey per bee but use less per bee over winter. Therefore, a bigger colony, having more foragers and in-nest bees, could allow a better observation of the effects of the pesticide because of the higher number of bees involved.

- However, there might be some advantages in using colonies of at least 10 000 bees instead of bigger colonies for the evaluation of the pesticides, but they do not seem to have been evaluated in the scientific literature. One of the advantages could be to assume that a 10 000 bee colony might be more sensitive to the tested pesticide than a bigger one, due to reduced resilience to replace foragers with nurse bees and a smaller brood area, and hence may have lower resilience to impacts. In an experiment

designed to evaluate the effect of population size of colonies of honey bees in field evaluation of stock (strain breeding), Harbo (1986) showed that the optimal colony size was 9000 bees in these experimental conditions, and that the optimal size for stock testing will vary with climate as well as season.

- More research should be made in order to evaluate the effects of the colony size in the field tests in particular with regard to the sensitivity to detect pesticide effects in both adult bees and brood.

5.4.2.2 Exposure routes (see also Chapter 3)

For the completeness of the field test, three exposures must be considered: contact, ingestion and inhalation (for spray application). Honey bees can be exposed through the nectar, the pollen and the water. These exposure routes must be analyzed for two categories of bees: foragers and in-nest bees. In all cases honey bee exposure should be demonstrated.

Before addressing exposure routes, it is important to analyze if experimental conditions in the field tests allow an optimal exposure of colonies, corresponding to the conditions encountered in natural situations.

5.4.2.2.1 Level of exposure of the colony

The foraging distances being variable between honey bees implies that the foraged surfaces are also variable. For example, an average foraging distance of 2.5 km corresponds to a theoretical foraging area of 19.6 km2, i.e., about 2000 hectares. Obviously this whole area is not available for foraging bees as it is not only composed of melliferous or polliniferous flowers. However in areas of intensive agriculture, at certain times of the year, honey bees can be exposed to large areas of crops treated with pesticides.

In the guideline EPPO 170, the surface of the test field is 2500 m² or a 1 ha. A field of 1 hectare represents 0.05% of the average foraging surface for bees and a field of 2500 m2 represents about 0.01%. For bees foraging at 5 km, the foraging surface will be around 80 km².

Whatever the level of exposure, one must acknowledge that, given the low surface of the test fields, as regards to the total food available for the colony, the quantity of nectar or pollen brought by honey bees having foraged in the test field (2500 m² or a 1 ha) could be small compared to the quantity of nectar already available in the colony (in the food stores) or brought from other sources that honey bees can visit during the experiment (beyond 50 or 100 m, up to several km).

Hence, the bees could be exposed to an unrealistically low total quantity of toxic substance, if residues are expected to be available in a large area at a similar time, e.g. in the case of SSST. This quantity will be much lower than that to which bees are exposed in real conditions, when the surface of all the treated fields in their foraging area will be significant (hundreds of hectares or more), and where the interval between the flowering periods for the different fields in the same area, can lead to exposure lasting for several weeks to more than a month.

It is essential to ensure alternative significant sources of forage, e.g. other flowering crops, are not readily accessible to the test colonies, e.g. by assessing the level of residues returning to the hive and confirming the sources of pollen by palynology.

5.4.2.2.2 Level of exposure of the foragers

a) Exposure by contact

i. Sprayed products

The exposure route of the foragers is covered for the nectar, pollen and water foragers.

ii. SSST products

The exposure route of the pollen foragers is covered.

The nectar foragers can be exposed during the nectar intake by contact with the mouth parts, the tongue, the oesophagus and the crop, which is identical to the nectar uptake in a spray application.

b) Exposure by ingestion

i. Energy expenditure

Actually, it is very difficult to precisely know the exposure of the foragers by ingestion, because these bees are carriers transferring the nectar to the colony where it will be transformed and stored for future use by the bees inside the colony. A bee flies from the hive with some sugar provisions in her crop, consisting of diluted honey or nectar. In addition to the sugar contained in the crop, we can infer from the scientific literature (Brandstetter et al., 1988; Gmeinbauer and Crailsheim, 1993; Visscher et al., 1996), that the honey bees leave their colony with a sugar reserve of between 1-2 mg in the bee muscles, hemolymph and midgut contents. It means that, in theory, they can fly around 2.1 - 4.2 km. Since the distance between the colony and the tested field is very small (50 to 100 m), the energy expenditure is extremely low for the bees to return to their colony, and probably they consume a very small quantity of this nectar, if any at all, and regurgitate the major part in the hive. However, Gary and Lorenzen (1976) reported that 5-10% of nectar of the crop may be involuntarily taken into the proventriculus during foraging. Taking into account this phenomenon, it is possible that a small part of the nectar collected by bees is metabolized, which would expose them to low doses of pesticides by ingestion.

ii. Foraging distances

Concerning the foraging distance of the honey bee, it has been shown that, in natural conditions, it can vary between some hundreds of meters until 10 km or more from the hive depending on the available forage (von Frisch, 1987). Bees have been shown to collect nectar up to 13.5 km from their colonies (Eckert, 1933). The foraging distance varies depending on the environment, the colony strength, the needs, the genetics of the colony, etc.

Several scientific publications (Visscher and Seeley, 1982; Beekman and Ratnieks, 2000; Steffan-Dewenter and Kuhn, 2003) have presented results on the mean, median and maximum foraging distances. These experiments have been identified following different evaluation methods, with colonies of different sizes, respectively 20000, 4000 and 4000 individuals. The results obtained are the following:

- median distance: 1.6 km, 6.1 km and 1.2 km
- mean distance: 2.2 km, 5.5 km and 1.5 km
- maximum distance: 10.9 km, 12 km and 10.0 km.

In the protocol of the field experiment following the guideline EPPO 170, the hives are placed on the edge of a field of at least 2500 m^2 (e.g.50 m x 50m field) in the case of a *Phacelia* crop, or on the side of a field of approximately 1 ha (e.g. 100m x 100m field) for other crops. In both cases, only a proportion of foragers will forage in this field. All the other bees may forage in other fields, beyond these test item fields (up to several km from the hive). Therefore, one cannot exactly know the proportion of exposed bees.

Even if the tested crop is attractive for honey bees, the population of foragers will distribute themselves on all the available flowering and attractive plants. If a field test is done, the experimenter should rigorously demonstrate that a significant number of foragers have been exposed to the tested pesticide through residue analysis of pollen and nectar returned to the hive.

iii. Sprayed and SSST products

The pollen foragers do not eat the pollen so they are not exposed by ingestion. Nectar foragers that collect the nectar in a field that is very close to their colonies have a low (or no) true consumption of collected nectar but the honey bee crop is leaky so 5-10% permeates into the ventriculus (intestine) (Gary and Lorenzen,1976).

c) Exposure by inhalation

i. Sprayed products

This route is covered because treatments should be applied when the test crop is in full blossom and during the daytime, when bees are demonstrated to be actively foraging on the test crop. Nectar and pollen foragers are exposed.

ii. SSST products

The exposure will depend of the vapour pressure of the compounds.

d) Conclusions concerning exposure routes for the foragers

The exposure of bees exposed by contact to a spray product is well identified. In all the other cases, and especially for the exposure by ingestion to sprays and to SSST products, uncertainties exist concerning the real exposure of foragers. These uncertainties are due to the unknown quantities of toxic substances consumed, which may be affected by the low energy demands in bees in the context of the guideline EPPO 170.

The small size of the treated field and the very small distances between the colonies to this field may lead to the underexposure of the experimental colonies to the pesticide, compared to their exposure when they are located further from a treated forage source. Further research is required to develop a detailed protocol to assess any chronic sub-lethal effects associated with ingestion of nectar and pollen, e.g., as regards the orientation capacities of bees foraging at long distances.

5.4.2.2.3 Level of exposure of the bees inside the colony

a) Exposure by contact

i. Sprayed products

The bees inside the hive are only exposed in the case of direct drift of the spray through the aperture of the hive, which may occur for hives placed along the edge of the treated plot.

ii. SSST products

The bees inside the hive are only exposed in case of drift of the dust of systemic pesticide through the aperture of the hive during the sowing operations, which may occur for hives placed along the edge of the treated plot.

b) Exposure by ingestion

The bees inside the colony belong to two main categories: the adult bees (workers, drones and queen) and the immature stages (eggs, larvae, nymphs). All these bees can, in principle, be exposed to the active substance by the contaminated nectar and pollen brought back to the colony by the foragers.

The scheme EPPO 170 under-estimates the exposure of the honey bee colonies in the test fields compared to those belonging to beekeepers, for several reasons:

- if the colonies contain significant reserves of non-contaminated pollen and honey that serve as food;

- the low surface of test crops (2500 m² or 1 ha) may not allow foragers to bring maximum quantities of toxic substance to the colony, particularly if there are other significant sources available. Some of the foragers (not quantified) will not visit the treated field, but fly beyond the test field to bring back nectar and pollen that have not been contaminated. These are stocked in the hive or consumed during the experiment;

- all or part of the contaminated nectar and pollen are stocked in the colony and not used immediately. One cannot know how much of it will be consumed during a relatively short duration study;

- one of the differences between non-systemic sprayed and the SSST products is that the non-systemic sprays are applied on the surface of the plant and are only brought to the colony over a limited period. On the contrary, the SSST products and the systemic spray products are present in the pollen and nectar for the duration of flowering, and will be continuously brought to the colony as long as flowering persists, and they will be stored in the hive and available over longer periods extending over the flowering period;

- the maximum duration of the experiment in the guideline EPPO 170 (28 days) is not adapted to assess the effects of systemic pesticides on all the categories of bees inside the colony. The life of a bee is about 50 days or more during spring and summer (21 days of pre-imaginal development and at least 28 days for the adult life), and several months in winter.

c) Conclusions concerning exposure routes for the in-nest bees

No detailed protocol is proposed for measuring the effects of the test pesticide on the lifespan of the different categories of in-nest bees, in particular the nurses tending the brood, or for measuring sub-lethal effects.

The exposure is not currently quantifiable for in-nest bees, because foragers bring back an undetermined quantity of nectar and pollen coming from untreated crops, present beyond the test fields.

Given these conclusions, the results obtained on behavior or bee disorders will have to be interpreted with caution. In particular, if no effect is seen, the experimenter should make sure that a significant number of honey bees from the tested colonies have really foraged in the experimental field, using nectar and pollen analyses both from returning foragers and from representative samples taken from within the hive. These can be compared with the residues collected from semi-field studies where bees are confined to the treated crop. For pollen, the experimenter should be sure that the proportion of pollen collected in the experimental field is predominant comparing to other crops visited (bearing in mind that different crops vary in attractiveness, e.g. *Phacelia* is highly attractive, maize less attractive).

5.4.2.3 Duration

a) Sprayed products

The test duration for sprays is sufficient for the analysis of potential foraging problems (from 1 to 7 days for foragers and once a week during 4 weeks inside the hive), but should be extended to two brood cycles (42 days) for the observation inside the hive to ensure that effects on development of brood, e.g. brood mortality, are included.

b) SSST products

The duration of the test for systemic products is insufficient for foragers and for honey bees inside the hive, and should be prolonged (see 5.4.3.1.3).

Concerning foragers, in agricultural environments, honey bees foraging on the same crop species can potentially be exposed to pesticides contained in the nectar and the pollen for long periods (several weeks), if there are large areas containing fields of the same crop which do not flower at the same time but consecutively, e.g. over several weeks (sunflower, maize,…). This point is especially important in cases where the available nectar and/or the pollen contain systemic insecticides which are returned to the colony continuously over several weeks.

Concerning honey bees inside the hive, given that contaminated nectar and pollen are brought to the hive by foragers and are stocked during a shorter or longer period before being consumed, a long-term colony monitoring must be done.

5.4.2.4 Endpoints

5.4.2.4.1 General remarks:

Unfortunately, in the document EPPO170, no detailed protocols are provided and therefore it is not possible to evaluate the robustness of any chosen parameters. In its actual form, it gives experimenters too much margin of interpretation. This is, by the way, several times mentioned in the guideline: "*It is important that this guideline is interpreted with appropriate flexibility*" [….] "*As with the semi-field tests, it is intended that this guideline should be interpreted with appropriate flexibility".*

For example:

- *"general behaviour of bees on the crop":* here we need precise information on the nectar and pollen gathered: which are the parameters to be used?
- *"general behaviour of bees around hives using a standardized approach":* which approach exactly, which parameters?
- *"colony status / development":* measured by which method?
- *"In some cases, according to the requirements of the study, it may also be appropriate to include additional assessments: pollen collection (e.g. by using pollen traps or by other appropriate methods); pollen and nectar storage; hive weight development; more detailed brood assessments; specific behavioural observations; and determination of residues in relevant bee and crop matrices (e.g. dead bees, nectar, pollen, wax and /or honey)":* this proposal is very interesting and should be systematically used for all studies.

In this case, again, the monitoring protocol should be precisely designed and some factors are discussed below.

5.4.2.4.2 Foragers

a) Sprayed products

The proposed experimental conditions seem to satisfactorily evaluate the flight and/or foraging activity in the crop, as well as the mortality in front of the hive.

b) Sprayed and SSST products

Some important endpoints can probably not be evaluated because of the short distance between the hives and the test crops. This is the case for orientation disorders in foragers. The orientation abilities allow honey bees to remember the environment of their hive in an area of several tens of km^2. If orientation is disturbed during foraging, the consequences will be very important because the honey bees will not find their way back to the hive and will die within a few hours (because they cannot survive outside the colony for a long time). However, in the field experiment where the colonies are placed in or on the edge of the flowering crop on which exposure will take place, the honey bee does not need all its orientation abilities to find its way back to the hive. Experimenters could miss the eventual sub-lethal effect on orientation abilities. Therefore additional tests must be made, particularly regarding homing flights to the hive after foraging at long distance (from hundred of meters to several km). Methods have been proposed in the literature and could be adapted (see 5.4.3.2). Research on this subject should be developed to enable robust protocols to be recommended.

5.4.2.4.3 In-nest bees

For SSST products: as described in 5.4.2.2.2, contaminated pollen and nectar may be consumed by all the categories of bees in the hive (including the brood) during a shorter or longer period. The protocol, as it is now, is not adapted to assess the endpoints specific to the categories of bees inside the colony.

5.4.2.5 Control, number of dose and replicates, statistics

a) Control (Sprayed and SSST products)

If there is overlap in the foraging area of the control and tested colonies, the results obtained cannot be validated. The distance between test and control colonies *("2–3 km depending on local conditions")* may be insufficient for preventing cross-foraging between treated and control plots. If there is an overlap in the foraging area of the control and tested plots, colonies cannot be excluded and therefore the results obtained cannot be validated. From the scientific papers cited above concerning the mean, median and maximum foraging distances, we can admit that an (unknown) part of the control bees can forage in the treated field, at 2.5 km. Consequently, if they go back to their colonies showing toxic effects, or if they die, this will reduce the difference of effects between treated and control bees, and will lead to underestimation of the toxic effects of the tested pesticide.

It is agreed that there may be variations in the climatic and landscape conditions between sites depending of the distance, but the EPPO 170 does not scientifically justify the choice of a minimum distance of 2-3 km. The scale of the climatic and landscape variations depends on the sites. In some areas, the weather and the landscape are changing rapidly and may preclude greater distances between plots whereas in other areas it happens over wider geographic scales. Therefore, to conduct field tests, it is proposed to choose areas presenting similar environmental conditions, where possible at least 4 - 6 km away apart. If smaller distances exist between plots it is essential that residue analysis of pollen and nectar is undertaken from foragers returning to the hive to confirm that bees from control colonies are not foraging on the treated plot.

As in any scientifically rigorous experimentation, the question of controls is crucial, because they provide a baseline for characterizing eventual effects. In field experiments, the control colonies must be placed in a comparable environment and should not show abnormal mortality.

b) Number of colonies per treatment/plot and replicates (Sprayed and SSST products)

The number of test and control colonies (four in the guideline by EPPO 170) must be high enough to account for the normal inter-colony variability and allow statistical analyses. As the inter-colony variability is often high, an adequate number of colonies of each modality (control and experimental) should be required.

The sentence "*Although very desirable, replication is often not feasible because of the requirements for separation*" is not acceptable. If replicates are needed, they have to be done. If this cannot be on the same area for reasons of separation, then it has to be done in another area (control and test colonies).

The number of replicates/controls should always be in relation to the magnitude of effect that should be detected in the test. An analysis of the statistical power to detect a certain magnitude of effect should always be provided for parameters assessed in the test, e.g. foraging activity, homing, mortality at hive or colony development.

c) Statistical evaluation (Sprayed and SSST products)

The sentence "*it has been recognized that statistical analysis may not be feasible*" is unacceptable. As in any scientific experiment producing quantitative data, statistical analysis must be done, which is generally not the case in current testing either. If it cannot be done, the reason must be that the number of data is too low. Therefore additional data must be obtained. This can be done, in the first place, by using a sufficient number of colonies. The number of measurements on foragers and on colony and brood assessments must be equally increased for allowing statistical analysis.

5.4.2.6 Conclusion

a) General conclusions

The comments above show that the application of the methods described in the guideline EPPO 1/170 (4)*, Side effects on bees, Field tests* must be improved, to more fully assess the effects of pesticides at the scale of the colony, including all bee categories and relevant long-term effects.

In particular:

- More detail is required in the description of methods. There is too much room for interpretation of methods by experimenters. More precise protocols must be developed and validated.
- The low number of colonies used is insufficient for grasping the important inter-colony variability characterizing honey bees.
- The distance between the control and the test fields may not prevent control bees from foraging in the test fields and being exposed to the test substance.
- Statistical analyses of results need to be compulsory.

b) Sprayed products

The test conditions for spray seem robust enough for detecting some eventual foraging problems. However, some parameters are insufficient for being certain of identifying potential effects, e.g. the foraging distance and the plot surface.

c) SSST

Because the protocol was originally intended for spray products, several shortfalls can be noted concerning the tests for systemic products; they have already been mentioned in the comments above on the life stage, exposure route, endpoint and duration.

It is therefore important and urgent to develop more precise guidelines, in particular for separating exposure to systemic insecticides used in seed and soil treatment from sprayed products.

5.4.3 Suggestions for improvements

5.4.3.1 Improvement of the EPPO 170 (4) guideline Field tests.

Several recommendations are provided below. They could serve in the development of rigorous protocols that are not currently included in the guideline EPPO 170, and which would allow a better control of the exposure and observation of the eventual effects. In all cases both the statistical robustness and biological significance (threshold of concern) of the proposed approach must be evaluated in determining the most appropriate method.

5.4.3.1.1 Colony

For testing a pesticide on a given crop, the most realistic conditions are to use colonies having the same level of development as the other colonies in this region at the time of year when they forage on the respective crop. The choice of using a small colony for the local conditions (e.g. 10 000 bees) in the field experiment in late spring or in summer should be scientifically justified.

The colonies should be healthy at the beginning of the experiment, e.g. free of clinical signs of significant brood diseases such as AFB and EFB. As most of the European colonies, even strong ones, contain infectious agents, it is not possible to use colonies that are completely free of them. Regarding the mite *Varroa destructor,* present in almost all European colonies, the level of infestation of the control and test colonies should be as low as practicable. It is very important that the health condition should be the same in control and test colonies.

During and after the experiment, the health of the colonies should be evaluated for the whole range of bee diseases (including *Nosema*, acarine and the main viruses, e.g. through molecular screening).

Indeed, it has been shown that low levels of some pesticides may have synergic actions with diseases such as *Nosema*. Finding diseases in test colonies, which were healthy before the experiment, and not finding such diseases in control colonies, can imply a synergic effect of pesticides and diseases.

5.4.3.1.2 Proposals for controlling and increasing the exposure of bees

It is important to demonstrate that foraging of colonies has occurred on the correct target crop, e.g. by use of highly bee-attractive crops with regular foraging assessments, and has not occurred on the matched field, e.g. by residue analysis of pollen and nectar in bees and from hives located on the control field.

In order to measure the proportion of pollen coming from the treated plants compared to pollens coming from other plants in the foraging area, pollen traps should be provided in some test and control hives, for further pollen analysis. This pollen analysis should not be limited to the observation of the pollen pellets colour, but should include the analysis of these pollen pellets under the microscope (palynology).

Residue analyses must be performed on the nectar and pollen brought back to the colonies in the tested and the control colonies. These analyses should have two goals: the first one, to check that the bees near the treated field have been exposed to the pesticide, and the second one to check that the control bees have not been exposed to the pesticide of the treated field or by another one, also present in the

environment. If there are residues detected in the controls and no effects detected compared to treatment, then the study is not valid. If effects were detected then the study could still be used as supporting information, but the study must be repeated if the conclusions of the risk assessment rely on it.

The test should allow the measurement of the exposure level through several methods, comparatively between test and controls. One should, at the same time, consider the foraging bees on the flowers and when they go back to the colony, and the in-nest colonies. The observations must be evaluated following both quantitative and qualitative approaches. As examples:

a) The foragers on the flowers

In order to control the level of exposure of nectar and pollen foragers, the foragers should be counted on the test and control crops, at different moments of the day, during a significant period of time, and during all the duration of the experiment. The number of data collected should be sufficient for allowing statistical treatment.
A qualitative approach should observe precisely the behavior of nectar foragers and pollen foragers using - but not limited to - behavioural effects identified in CEB 230 (CEB, 2003).

b) The foragers on the flight back to the hive

A qualitative approach should observe the behavior of the bees on the hive's flight floor, in particular eventual aggressive interactions with the guard bees.

c) The in-nest bees

High mortality of young adults (especially if they can be distinguished from foragers), unusual mortality of any brood stage (larvae/pupae might be seen in dead bee trap) or the disappearance of the queen, eggs or larvae from the hives located at the treated field are all indications that in-hive bees had been affected by the test item.

To ascertain that the hive bees and brood (larvae) are exposed to the contaminated food that had been carried in the hive from the treated area, it is necessary to evaluate the storage and the consumption of pollen and nectar during the study (see below: d). It is important to reduce excess stored pollen and nectar (see below: e) at the start of the exposure period to a level to limit dilution of forage from the experimental plots. Assessment of effects may include:

i. quantitative approach

Concerning brood development and brood mortality there is specific brood development methodology; e.g. the OECD 75 guideline, which can be used in the field. Colony level assessment of brood development can be used to monitor the areas of eggs, young larvae and sealed pupae over time (e.g. "Liebefeld method", see Wille and Gerig, 1976a,b,c; Gerig, 1983; Imdorf et al., 1987). Also dead bee traps may provide some information for larva. Consideration may be given to the use of a thermal probe which can be used for following the evolution of the brood temperature but the use of this as a robust measure of effect is required.

ii. qualitative approach

In order to observe the behavior of in-nest bees, and in particular the nurses, and the sub-lethal effects inside the hive, protocols should be precisely defined in future work.

iii. residue analyses

Residue analysis of nurse bees/dead bees from dead bee traps especially if it is apparent that it includes (young) nurse bees. Residues found in nurse bees demonstrate consumption of contaminated food and/or feeding of larva with contaminated food.

Residue analysis of young pupae. Residues found in pupae demonstrated that the larvae had been feed with contaminated food; samples of pre-pupal larvae are preferred not earlier than 6 days after the treatment/start of the test, preferably at later and more than one sampling times.

d) Evaluation of the storage of food

Follow up of the storage of the food items with in-hive observations (i.e. when the strengths of the colony or brood development is controlled) can be useful for more exact estimations of the exposure.

The methods suggested for controlling the brood development (e.g. "Liebefeld method", see Wille and Gerig, 1976a,b,c; Gerig, 1983; Imdorf et al., 1987) might also be used to estimate the extent of the surface area of the comb(s) containing food. The simply visual assessment of each comb is the preferred method. The percentage of the area of the food items (such us pollen, nectar, beebread or honey) should be estimated knowing the total comb area of the hive. Alternatively, a net of squares or oblongs (raster frame) can be placed loosely in front of the honeycomb and the number of units (squares or rectangles of the raster) filled up with the items is estimated.

Another approach is using transparent sheets (placing them in front of the comb, one for each side) where the exact place and the size (with the contour) of the pollen or nectar patch(es) is/are marked.

A methodology using digital photography to control the brood development has been developed (Jeker et al., 2012), which might also be a useful tool for quantitative assessment of the stored food items on honey bee combs. However, it is noted that the latest two methods might considerably disturb the bee colony (i.e. shaking the bees from the frames) too often during the study.

If one of the methods described above is used at each in-hive observation, the change (decrease/increase) in stored food can be followed throughout the study. These data might allow a conclusion on the storage of contaminated food and on the food consumption by the colony. The shortcomings of these assessments only provide indirect indication of the consumption of contaminated food and the exact amount cannot be established. Also, in case of field studies or bee brood feeding studies the dilution rate is not known. However in case of a semi-field study for the period when the colony is confined, the only source of fresh pollen and nectar is the test area.

e) Removal of the food stocks

In order to reinforce the level of exposure of bees to the contaminated nectar and pollen, most of the frames containing food stocks could be removed from the colony before the beginning of the experiment to a level that just prevents starvation. But this operation is risky and should only be conducted by experienced beekeepers because it could cause a weakening of the colonies, and it should be minimised to allow sufficient stores for survival.

Nevertheless, this method cannot prevent foragers who do not visit the test field from bringing pollen and nectar that are not contaminated to the hive. The proportion of contaminated or not contaminated food brought to the hive can be monitored by measuring the residues both being returned to the colony by foragers and within the colony with appropriate LOD and LOQ.

5.4.3.1.3 Duration

The colonies used in experiments (including controls) should be monitored for a longer time covering all the flowering period and beyond. The study should last at least 2 brood cycles to ensure a significant proportion of brood is exposed to residues stored within the colony.

As the contaminated honey and pollen stores could be consumed during winter (honey) and after the wintering (honey and pollen), where residues are persistent, monitoring should be maintained until this period. The technical protocol of this monitoring should be precisely designed.

The proposed follow-up of the test colonies should take into account the persistence of the pesticide residue in determining whether long-term effects should be included. For the long-term study, including the over-wintering, the test and control colonies should be placed in an area far from fields in intensive agriculture in order to limit to the maximum their exposure to pesticides. If they are exposed to pesticides during this period, the control colonies could be also affected. This exposure could reduce the difference of effects between treated and controls, and will lead to underestimation of the toxic effects of the tested pesticide.

5.4.3.1.4 Control, number of dose and replicates, statistics

Some proposals concerning the question of the control colonies have already been made above.

- The control colonies must be placed at a distance of at least 4 - 6 km from the experimental field, because this distance reduces the exposure of the control colonies to the tested pesticide. A shorter distance (in the EPPO 170: 2-3 km) may permit cross-foraging between treated and control plots, although probably to a lower extent.

- Analyzing the evolution of the daily mortality and colony development for one to two weeks before the exposure period would produce useful data to be used as additional reference for the experiment. In this case, each colony will be its own internal control. The two series of control data (internal and external) would insure the best conditions for detecting the potential effects of a pesticide. The crop should flower for a mínimum of 2 weeks (although this is dependent on the proposed crop use) in order to have an optimal condition for the spray application to maximise exposure. For the SSST the duration of the exposure could be longer in relation to the crop (maize, sunflower, rape…).

5.4.3.1.5 Conclusion

As no toxic standard is used, it is crucial to show that the honey bees forage in the crop (see above for proposed methods):

- in order to be sure that the colonies near the treated field and the control field will forage mainly on these fields it should be checked that no significant areas of flowering crops are present within the immediate foraging areas of these colonies, and especially of the same crop (4 km radius).

- in order to evaluate the exposure of the honey bees in the tested crop in relation to the other possible flowering crops in their foraging area, the floral origin of the pollen and the nectar brought back to the colony has to be determined by palynological and melisso-palynological analyses. If the level of nectar and pollen of the non-tested crop exceeds a certain threshold (research needed to define this threshold) this should be taken into account in evaluating the validity of the study.

- in order to avoid potential overlaps in the foraging area of bees from colonies near the contaminated and the control fields, it is proposed to increase the distance between these fields from 2 - 3 km to around 4 - 6 km, in an area where the microclimate and the environment areas are as similar as possible.

- the size of the test and control fields proposed in the EPPO 170 guideline are much lower than that of the average foraging area of honey bees, in order to provide better insurance that the exposure is effective and close to the real conditions encountered by bees. It should be recommended to use post-registration monitoring studies to cover this scenario.

5.4.3.2 Necessity to develop other methods

The objective of the present report is not to propose detailed protocols allowing the assessment of all the possible effects of pesticides on honey bee colonies in the field. An in-depth study still needs to be done for developing robust protocols taking into account the methodological limits inherent to the bee biology and behavior.

Concerning sub-lethal effects on honey bee behavior, or colony survival and development, some proposals are made in the scientific literature and below. See, for example:

- Review articles: Thompson, HM. (2002), Pham-Delègue et al. (2002), Desneux et al., (2007).
- For homing flight and orientation: Vandame et al. (1995), Bortolotti et al. (2003), Colin et al., (2004), Karise et al. (2007), Yang et al. (2008), Giffard and Mamet (2009) and Decourtye et al. (2011)
- Foraging behaviour: Bortolotti et al. (2003), Colin et al. (2004), Karise et al. (2007), Yang et al. (2008), Giffard and Mamet (2009).
- Fecundity, growth and development: Pettis et al. (2004) and Dai et al. (2010).
- The guideline CEB 230 (CEB, 2003) also gives some guidance on what behaviour should be looked at, but this should not be considered to be an exclusive list and if there are other effects observed they should also be reported.
- Concerning the evaluation of the effects on honey bee larvae, the OECD 75 guideline could be adapted for use in field studies.

5.4.4 When to use field tests

Field tests are necessary for assessing the global activity of honey bees and the good functioning of colonies, in conditions close to normal situations. Indeed, semi-field tests being done in confined conditions, on very small treated surfaces, and during a short time, cannot reflect natural conditions where honey bees forage on very large surfaces, during long periods.

However, the EPPO 170 guideline presents numerous limitations, in particular related to understanding it's ability to realistically replicate honey bees' exposure (see 4.4.2.2).Trying to observe all the effects on a colony using a single generic test is unrealistic because of the complex social life of honey bee colonies, which can be considered as superorganisms.

For this reason, two complementary types of field tests should be done (potentially as subsets of the same field study) when a pesticide is suspected of toxic effects:

- a global test, based on the principle of EPPO 170 guideline but with further improvements (see 5.4.3.1)
- several tests for assessing the effect of a pesticide on certain particular behaviors, such as, for example, the homing flight and orientation, the foraging behavior, etc. (see 5.4.3.2). It is therefore important and urgent to develop robust guidelines for quantifying and interpreting these sub-lethal effects.

5.4.5 Field test for social non-*Apis* (bumble bees) and solitary bees

5.4.5.1 *Bombus* spp.

Several approaches have been used to assess the effects of applications of pesticides on bumble bee colonies in the field including confining bumble bee colonies in large cages to the treated area (Gels et al., 2002) and open field studies with study colonies (e.g. Schäfer and Mühlen, 1996; Tasei et al., 2001). Foraging activity of wild bumble bees on open plots to determine if insecticide treated areas were avoided was reported by Gels et al. (2002).

In general a similar approach to that used for honey bees has been adopted in the small number of published open field studies. Six to ten small bumble bee colonies were placed in a treated field (2-16 Ha) and a control field. Assessments of effects included colony vitality (i.e., numbers of brood, workers, and honey pots, and weights of queens, workers and whole colonies with hives), workers foraging activity, marking all introduced workers to assess homing rate and growth rate of the colony, and defensive response to an aggressive stimulus. Pollen and nectar sampling for residue sampling and assessment of forage was undertaken by collecting foragers returning to the colonies (Tasei et al., 2001). Significant further work is required to develop guidelines, including the minimum field size, number of colonies per treatment, methodology for dead bee assessments and foraging assessments and agreement of appropriate approaches for determining colony development.

Further work is required to assess the effects of pesticides on bumble bee colonies through the standardization of routine procedures to assess the effects of pesticides at the colony level. Due to the structure of the nest the only available methodology currently is to kill the colonies at the end of the study and assess the numbers of eggs, larvae, pupae and adults. Current methodology does not allow assessment of the starting colonies for comparison over time. An additional concern in field studies is that only endemic species and sub-species are used so as to not introduce competing species/sub-species to the environment; this issue also applies to bumble bee diseases as bumble bees used in toxicity tests and in pollination are not routinely screened for disease.

5.4.5.2 Solitary bees

A few studies have reported field tests on solitary bees and they are limited to the alfalfa leafcutting bee *Megachile rotundata* (Torchio, 1983; Mayer et al., 1998). In the earliest one the author placed two nesting shelters in two distant parts of an alfalfa field (1 km x 45 m). One half was treated and the second half was used as control. Fifty nests were marked and monitored in each part of field before and after the treatment. The active nests were examined nightly and the daily number of nesting bees and the daily cell production rate were assessed. When the nesting period was completed the marked nests were dissected to record larval mortality. In another study the exposure of foragers was estimated by analyzing pollen sample from brood cells provisioned by nesting females before and after treatment (Tasei and Carre, 1985). Due to the smaller foraging range of most solitary bees, the field tests are suitable to study the effect of pesticides in outdoor conditions.

However, further work is required to develop guidelines, including the optimal field size, the minimum distance between treated and untreated plots and the number of females released per treatment.

Since the rearing methods may affect the performance of nesting females, an agreement on the methodologies of how to manage solitary bee populations is required to standardize the available protocols.

- The strength of a scientific approach is given by the fact that precise protocols are respected, which guarantee the reproducibility and the reliability of the results obtained.

- Recommendations for certain endpoints in a future scheme have to be followed by the experimenters. It would be the notifier who needs to justify if certain endpoints are not observed.

- Training may be needed for persons conducting bee studies because observations of behavior in bees are hard to see by non-experienced personnel. The experimenter should be able to explicitly prove either his/her training and practice as an experienced beekeeper.

- Training is also very important for those who evaluate the tests for regulatory purposes

5.5 Recommendations for future research

Research aimed at developing tests for studying the toxicity of pesticides for bees:

- at individual level: chronic and sub-lethal toxicity for adult workers, effects on the fecundity and longevity of the queens and fecundity of the drones, effects on the development of pre-imaginal stages (eggs, larvae and nymphs) including brood feeding and heating.

- at the colony level: chronic and sub-lethal toxicity, long-term effects studies

Precise and replicable protocols[1], and methods appropriate for statistical evaluation, should be developed for laboratory, semi-field and field testing. Criteria for checking the quality of laboratory, semi-field and field testing should be established.

Concerning honey bees (*Apis mellifera*) including about ten subspecies (= geographic races) in Europe, inter-subspecies variability of the pesticides' effects should be studied, at least for those races that are the most used by European beekeepers.

Appropriate exposure scenarios and modeling approaches should be developed.

5.5.1 Laboratory

5.5.1.1 Prolonged exposure

- Development of standardized protocols for measuring the effects of prolonged exposure.

- Evaluation of the data generated by prolonged exposure studies in adults based on intermittent *versus* continuous exposures, in order to determine the most appropriate test design.

5.5.1.2 Sub-lethal effects

- Development of standardized protocols for measuring sub-lethal toxicity on the behaviour, physiology and neurophysiology of bees. The protocols should make specific reference to variability factors such as the age of the tested bees, the laboratory conditions (i.e. temperature and hygrometry), the bee subspecies, their nutritional and health status, etc.

- Research on the relationship between effects on individuals and effects on the colony should be done.

[1] For a review look at: Haynes 1988; Thompson 2003; Desneux et al. 2007; Decourtye and Devillers 2010.

- The relative sensitivity of test methods should be measured and linked to endpoints at the first tier on the colony/forager level effects (e.g.: PER, homing behaviour and *Bombus* microcolony studies).

- Suitability of laboratory based *Bombus* micro colonies for evaluating reproductive effects of products should be checked, both in their extrapolation to *Apis* and solitary bees and how to integrate the results into a risk assessment scheme.

- Development of molecular markers of toxic effects to enable prediction of potential sub-lethal effects such as immune system changes, potential interactions between products and effects in colony under real use conditions.

- Further study of the histological modifications caused by pesticides on bees to enable prediction of potential sub-lethal effects under real use conditions

- Determine thresholds that induce shifts in bee activity (e.g. sub-lethal effects induce a shift of nurses into foragers). Model and make scenarios on all the possible cascade effects on the colony.

- Analysis of interactions and synergisms (e.g. agrochemicals and veterinary products with diseases) at low doses.

5.5.1.3 Larvae toxicity tests

- Study the behaviour and physico-chemical characteristics of different toxic standards used in larvae toxicity tests in order to optimise exposure.

5.5.2 Semi-field

- Update the current protocols in accordance with state-of-the-art scientific literature on honey bees, for including precise descriptions of the behavioral and other sub-lethal and delayed end-points, for the individuals and for the colony.

- Specific guidance on the assessment of different behavioral endpoints should be provided, in particular concerning brood development and colony reproduction.

- Develop methodologies adapted to pesticides applied as SSST.

5.5.3 Field

- Development of standardized protocols for:

    i. a global test, based on the principles of the EPPO 170 guideline but with further improvements.

    ii. some specific tests for assessing the effect of a pesticide on specific behavioral endpoints, such as, for example, the homing flight and orientation, the foraging behavior, etc..

- Precise protocols should be developed for field testing of SSST products. These protocols should include precise descriptions of the behavioral and other sub-lethal and delayed end-points, for the individuals and for the colony.

- Automatic methods for measuring the activity of honey bee colonies should be developed or improved, e.g. for measuring the number of honey bees leaving the colony for foraging, the

number of foraging honey bees getting back to the colony and (by calculating the difference) the number of foraging honey bees eventually lost outside the hive.

- Comparison should be done among the sensitivity to a given pesticide of small (10,000 individuals) colonies versus normal colonies (30,000 – 40,000 individuals).

- Protocols for post-marketing studies to monitor the effects of registered pesticides should be developed.

6 CHAPTER 6: HOW TO TAKE ACCOUNT OF CUMULATIVE AND SYNERGISTIC EFFECTS

6.1 Summary

Pesticides containing a number of active ingredients are frequently applied sequentially or as mixtures such as tank mixes, and there is a consensus in the field of mixture toxicology that the customary chemical-by-chemical approach to risk assessment is too simplistic. This chapter aims to review the evidence on cumulative and synergistic effects of pesticide mixtures in bees and to develop recommendations for risk assessment purposes.

In terms of cumulative risk assessment, there is evidence that concentration addition is a conservative method and in previous reviews, the estimated toxicity using this approach was concluded to be more conservative than that predicted by independent action. Generally, at sub-lethal doses, exposure concentration addition has been observed more often than synergistic or antagonistic effects for mixtures of pesticides with a common mode of action and independent action (response addition) has been observed for compounds with a different mode of action. In some cases synergistic and antagonistic effects have also been observed and can involve two types of interaction: toxicokinetic and toxicodynamic interactions. Toxicokinetic interactions at the level of the absorption, distribution, metabolism and excretion can result in a decrease (synergistic) or an increase (antagonistic) in metabolism or overall elimination of the compound and may affect toxicodynamics. Toxicodynamic interactions can result in increase (synergistic) or decrease (antagonistic) in toxicity.

In order to develop methodologies to take into account cumulative and synergistic effects of pesticides in bees, the toxicokinetic and toxicodynamic aspects of pesticide mixtures in bees was reviewed. 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 i.e cytochrome P-450, glutathione-S-transferases and carboxyesterases. A number of studies have shown synergistic effects of pesticides and active substances applied in hives as medicinal treatments against *Varroa* mites in honey bees, for which toxicokinetic interactions were most commonly involved. The mechanisms of such interactions involved inhibition or induction of either detoxification enzymes (cytochrome P-450) or transporters which then enhance the toxicity of the mixture and decrease the LD50. There is also a growing body of evidence that there may also be interaction between pesticides and honey bee disease (fungi, bacteria and viruses).

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.

In the case of synergism which can be predicted based on the mode of action of the chemical classes involved (e.g. azole fungicides and insecticides), and in the absence of existing data on toxicity of the mixture, it is recommended to design full dose-response studies in adult bees and larvae for mixtures of potential synergists. These should take into account the dose dependency of the synergy, the magnitude of the interaction at concentrations of environmental relevance as well as both the maximum potentiating factor of the synergist and the concentrations for which no potentiating factor occur in the dose response curve. Such statistically sound dose response data will provide a basis to derive benchmark doses and their limit as suggested by EFSA's scientific committee. This flexible approach would allow quantitative protection goals to be achieved (e.g. specific effect size for lethality or for a sub-lethal effect depending on the protection goal and the aim of the risk assessment). Further work is also required to identify the molecular basis of interactions between environmentally realistic exposure to pesticides and the range of honey bee diseases (fungi, bacteria and viruses) to determine whether and how these may be included in risk assessment.

6.2 Introduction

In the environmental risk assessment of plant protection products, normally only the active ingredient or the formulation/product is taken into account. When the formulation contains more than one compound toxicity tests with the formulation have to be made available for the dossier (often only available for the most sensitive test species). In that case it is possible to compare the outcomes and to assess whether one of the compounds in the formulation behaves like a synergist.

When a compound or formulation/product is applied more than once in the growing season, the number of applications is taken into account in the registration process. However, only when the label of the formulation/product mentions that the formulation/product is used in a tank mix is the overall toxicity of the tank mix calculated on the basis of the dose (concentration) additivity; the tank mix as such is never tested. Note that in the tank mix not only could different compounds be mixed, but also additives, like for instance stickers and synergists, that will enhance the performance of the mixture.

The use of other pesticides in the same crop/field in the growing season or on neighbouring crops/fields are not taken into account in the environmental risk assessment. Organisms living in or close to such a field can be exposed many times (sometimes between 10 and 20 times) to one or more compounds (up to 4 is not an exception, see Chapter 2). In addition other compounds may be encountered in one of the environmental compartments. For instance, in the surface water, because neighbouring farmers or farmers upstream have used other compounds at the same time or during the same week. Vapour drift can also occur over relatively long distances from the source where a plant protection product was used.

6.3 Type of mixtures

6.3.1 Tank mixing

One of the few studies dealing with the contents of tank mixes was published by Fryday, Thompson and Garthwaite in 2011. The results of this study are summarized for 4 different crop types (e.g. arable crops, vegetable crops, orchards and soft fruit) in Table 6.1.

Table 6.1: Summary of applications for four different groups of crops

Crop type	Compounds in mixture	Mean a.i. per application	Mean a.i. in mixture	Unique combinations	% of total treated area	Year
Arable	2-9	3.26	6.15	5992	66	2008
Vegetable	2-7	1.49	2.81	1519	53	2007
Orchards	2-8	1.64	3.09	1099	60	2008
Soft fruit	2-6	1.58	3.24	891	46	2006

This shows that applications to 66% of the treated arable crop area contain an average of 6.15 compounds per application. For the other three crop types approximately 50% of the treated area is on average treated with three different compounds per application.
These data show that the use of tank mixes in agriculture is a common phenomenon.

6.3.2 Sequential exposure

Wildlife may not just be exposed to mixtures of compounds due to tank mixes. There is also the possibility that wildlife will be exposed to mixtures of compounds following sequential applications to crops or as they move between treated fields.
Research carried out in the Netherlands gives indications that wildlife living in or nearby a particular crop can/will be exposed either many times to the same compound and/or to many different compounds within one growing season (Spruijt et al., 2010; Luttik et al., in prep.). In Table 6.2 a

number of standard crop scenarios and in Table 6.3 a number of realistic worst case scenarios are presented.

Table 6.2: Summary of applications within one growing season for 11 crops (taken from Spruijt et al., 2010)

Crop (Standard scenario)	Number of times compound has been applied (n)	Fungicides	Insecticides	Herbicides	Others
Strawberries 2006	26	12	4	8	2
Asparagus 2006	16	6	5	5	-
Consumption potatoes 2008	23	18	2	3	-
Hyacinths 2008	29	8	11	10	-
Narcissus 2008	15	9	-	6	-
Leeks 2008	24	9	8	7	-
Sugar beet 2008	10	1	-	9	-
Tulips 2008	33	8	10	15	-
Winter carrots 2006	13	5	4	4	-
Winter wheat 2006	9	4	1	4	-
Seed onions 2008	26	14	3	8	1

Table 6.3: Summary of applications within one growing season for 6 crops (taken from Luttik et al., in prep)

Realistic worst case crop scenarios	Number of times compound has been applied (n)	Fungicides n + number of compounds	Insecticides n + number of compounds	Herbicides n + number of compounds	Others n + number of compounds
Fruit 1	52	35 (9)	11 (8)	6 (5)	-
Fruit 2	48	32 (10)	9 (8)	7 (4)	-
Tuber 1	21	11 (4)	7 (2)	3 (3)	-
Flower 1	36	8 (3)	14 (3)	14 (5)	-
Flower 2	82	40 (5)	29 (3)	18 (5)	-
Flower 3	52	22 (5)	15 (6)	15 (3)	-

In Figure 6.1 the sequential use of plant protection products is shown for the flower 2 realistic worst case scenario (according to Luttik et al., in prep). All applications are applied in a period of 26 weeks.

This information clearly shows that wildlife can be exposed to a multitude of compounds several weeks in succession.

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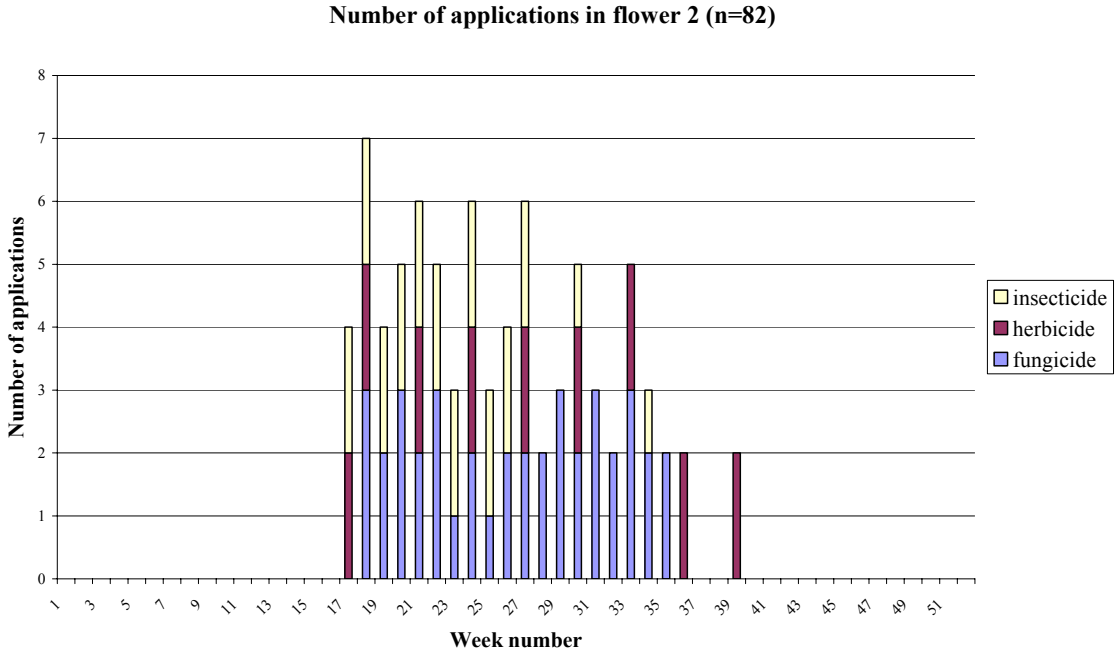


Figure 6.1: Sequential use of plant protection products

6.4 State of the art of mixture toxicology

There is a consensus in the field of mixture toxicology that the customary chemical-by-chemical approach to risk assessment might be too simplistic. There is a real possibility of underestimating the risk of chemicals to the environment. In binary and multiple mixtures of pesticides, most often concentration addition (CA) has been observed at low dose of exposure for compounds with a common mode of action (MOA) or independent action (IA) (response addition) for compounds with a different MOA. 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 (see paragraph below). 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.

Toxicokinetic interactions may cause deviations from additivity between the components of the mixture

1) Absorption and distribution: a substance (B) in a mixture may compete with the absorption of another substance (A) in the mixture or inhibit active transport or efflux pumps and affect the circulating levels of substance A (internal dose) in the body.

2) Metabolism and excretion: typical examples of such interactions include inhibition or induction of metabolising enzymes (such as cytochrome P-450) by chemical components of the mixture. Taking a binary mixture, with chemical A acting as a competitive inhibitor of the metabolism of chemical B, the elimination of chemical B might be slower (decrease in clearance, increase in half life of the chemical). Consequently, a change in elimination patterns may modify the patterns of the delivery of the dose of the chemical to the target organ and can potentially increase the toxicity of the mixture.

Toxicodynamic interactions involve interactions between the biological responses from exposure (internal dose) to the individual substances in the mixture. A typical example are interactions of chemical sharing similar biological targets (e.g., ligand-receptor interaction) or MOA such as triazole fungicides in mammals assessed by the PPR panel (EFSA, 2009a).

In a recent review for the European Commission (Kortenkamp et al., 2009), the use of the concentration addition model was proposed as the concept of mixture toxicity that is most relevant for hazard characterisation and ultimately can be integrated into the legislative process for risk management purposes. The use of the concentration addition has also been discussed by Verbruggen and van den Brink (2010). There are two reasons that make the use of this model concept attractive for policy makers. First, the model concept is generally more conservative than the concept of response addition. Nevertheless, the magnitude of the differences at low levels of exposure between the two models is usually small and hence, the outcome will not be overly conservative. A second reason for the use of concentration addition is that the model concept can make use of existing data such as a NOEC, EC10 or EC50's by applying the concept of toxic units (TUs).

The concept of TUs has been recently reviewed by the three non food committees of the European Commission (the Scientific Committee on Health and Environmental Risks (SCHER), the Scientific Committee on Emerging and Newly Identified Health Risks (SCENHIR), the Scientific Committee on Consumer Safety (SCCS)) which defined TUs as "the ratio between the concentration of a mixture component and its toxicological acute (e.g. LC50) or chronic (e.g. long-term NOEC) endpoint". In addition, the toxic unit of a mixture (TUm) has been defined as the sum of TUs of each individual chemical of that mixture. The committees also noted that the TUs concept only refers to a specific organism representative of a group of organisms ecologically or taxonomically relevant for the ecosystem (e.g. algae, daphnids and fish for the freshwater ecosystem) but not to the ecosystem as a whole (SCHER/SCENIHR/SCCS, 2011).

In practice, TUs can be used to quantify the toxicity of a mixture (assuming the dose/concentration addition principle) based on its composition. For instance, an acute lethal TUm of 10 would mean that a dilution of 10% of the mixture would produce 50% of lethality. If the slope of the concentration/effect curve is known, the TUm can be used to estimate the expected effect. The applicability of the TUs are currently-limited for the response addition model since it would require full dose-response relationships for all species and all compounds to be assessed and such detailed toxicological data is not usually available.

The application of TUs to environmental concentrations (EC) (predicted PECs, or measured, MECs) has been compared with the Hazard Quotient (HQ) approach used in human risk assessment. However, two major differences were noted: (1) HQ are then added into Hazard Index (HI) instead of TUm, (2) TUs refer to the ratio between exposure and a toxicological endpoint whereas HQ refer to the ratio of exposure to a Reference Value (RV) such as a Tolerable Daily Intake (TDI) derived using uncertainty factors from the toxicological endpoints (i.e. No Observed Adverse Effect Level (NOAEL), Benchmark Dose and its limit (BMD and BMDL) (SCHER/SCENIHR/SCCS, 2011; EFSA, 2009a).

Generally, the RV in ecotoxicology is the Predicted No Effect Concentration (PNEC), so that the sum of PEC/PNEC ratios could be assumed as comparable to HI since PNEC are derived by applying uncertainty factors to the toxicological endpoint in the most sensitive species which may be different depending on the chemical. Because of such species differences, PEC/PNEC for component of a complex mixture were concluded to be non homogeneous and cannot be added (SCHER/SCENIHR/SCCS, 2011).

Besides the toxic unit approach, other applications for the concentration addition concept can be used like the Toxic Equivalent Factor (TEF) approach, the Hazard Index (HI), the Point of Departure Index (PODI) and the Relative Potency Factors (RPF). These approaches are described in the EFSA/PPR

opinion on cumulative and synergistic risks from pesticides to human health (EFSA, 2008a; Kortenkamp et al., 2009).

A promising new development in the field of mixture toxicity is the modelling of the effects of sequential exposure instead of simultaneous exposure. A model that can be used for this purpose is the Threshold Damage Model of Ashauer et al. (2007). This model describes the cumulative (acute) toxicity of compounds that are not used simultaneously but sequentially, which is a common feature in agriculture. The use of such a model is currently limited, since the parameters describing toxicokinetics and toxicodynamics are only available for a few species.

Another important development is the application of species sensitivity distributions (SSD) in the field of mixture toxicity. This method (Posthuma et al., 2002), which is often referred to as multiple-substance potentially affected fraction (ms-PAF), calculates the percentile of species affected by the exposure to multiple substances at the same time (this is also applicable to concentration addition and response addition).

Finally, the concept of MOA is promising for mixture assessment in ecotoxicology, however, its applicability may differ from the MOA used in human risk assessment. Indeed, the three non-food committees of the EU further discussed the potential differences in the relevance of endpoints for human risk assessment versus ecological risk assessment. Ecotoxicological end-points are broader than endpoints used in human risk assessment since they relate to ecologically-relevant parameters (i.e mortality, fertility, reproductive capability). In contrast, in human RA, there are some effects such as molecular markers of carcinogenicity which are important for individuals of the population but would have negligible relevance in ecotoxicology. Importantly, in ecotoxicology, knowledge of the toxicological MOA on all the different types of species that may be present in an ecosystem is very much incomplete. This is well exemplified for pesticides with MOAs that are well characterised in target organisms but scarce for non-target organisms. In this case, pesticides may target a particular physiological or metabolic function that may not be common to all species in the ecosystem especially for species that are far taxonomically and for non-target organisms the effect of the chemical is likely or often assumed to be of the narcotic-type (baseline toxicity). For example, organophosphate and chlorinated insecticide toxicity in algae is "baseline", narcotic-type whereas triazines toxicity are a magnitude higher since they inhibit photosynthesis. Hence, "common MOA" in ecotoxicology may refer to broad end-point (reproduction impairment, population growth, mortality, etc.) (SCHER/SCENIHR/SCCS, 2011).

6.5 Examples of synergistic effects in ecotoxicology

Examples of synergistic effects of pesticides in invertebrates are presented below in aquatic and terrestrial organisms and finally in bees to give the reader a broad perspective of work conducted to date.

6.5.1 Examples of synergistic effects between pesticides in aquatic organisms

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 (see Table 6.4).

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

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 on

zooplankton 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. Authors discussed ways to tackle the question of synergy between chemicals in environmental risk assessment and proposed two approaches:

1) Testing maximum potentiating factor of proposed synergists towards high risk chemicals such as pyrethroids, to determine the size of extra uncertainty factors to be added to the pesticides having the synergy and including more sensitive species in tests

2) Investigate within the dose response curve, the dose at which no potentiating factors occur between the compound tested and the synergists (Bjergager et al., 2011).

6.5.2 Cumulative and synergistic effects of pesticides in insects and invertebrates other than bees

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. Authors highlighted that these results show that deviations from concentration addition can happen even with similar acting compounds, but that the nature of such deviations are species dependent and concluded that the concentration addition model may need to be used in a probabilistic context, rather than in its traditional deterministic manner (Gomez-Eyles et al., 2009).

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. The authors concluded that detailed analysis of toxicokinetics and toxicodynamics can aid further understanding of interactions in mixtures (Svendsen et al., 2010).

Finally, synergistic effects of insecticides (bifenthrin, imidacloprid) on tawny mole cricket (*Scapteriscus vicinus Scudder*) adults and nymphs, have been shown by injecting 5 µg per insect of each compound either as single compound or a binary mixture. Bifenthrin and imidacloprid provided the fastest median mortality causing immediate knockdown of the insects when injected and 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. The authors concluded that the combination of the sodium channel toxin (bifenthrin) and the synaptic toxin (imidacloprid) lead to the synergistic effects, which to the authors'

knowledge provides the first documented evidence of synergistic neurological ‘‘potentiation’’ (Kostromytska et al., 2010).

Natural conditions resulting from the interaction between "natural" and chemical (anthropogenic) stressors can have dramatic effects on environmental species and such effects were recently reviewed for more than 150 studies and included stressors including heat, cold, desiccation, oxygen depletion, pathogens and immunomodulatory factors combined with a variety of environmental pollutants. Overall, 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).

6.5.3 Cumulative and synergistic effects of pesticides and active substances applied in hives as medical treatments in bees

Bees have specific features in their genome and at the level of detoxification enzymes that influence the toxicokinetics of pesticides. These genetic and metabolic particularities in bees are briefly summarised below.

6.5.3.1 Metabolic and toxicokinetic particularities in bees

Recent sequencing of the honey bee *(Apis mellifera)* genome has revealed that it lacks DNA methylation genes, major transposon families, genes for innate immunity and detoxification enzymes, cuticle-forming proteins and gustatory receptors. In contrast, *Apis mellifera* genome has more genes for odorant receptors, and novel genes for nectar and pollen use consistent with its ecology and social organisation (Johnson, 2008).

In terms of detoxification enzymes, honey bees possess only about half as many glutathione- S-transferases (GSTs), cytochrome P450 monooxygenases (CYP) and carboxyl/cholinesterases (CCEs) compared to other insects. This includes 10-fold or greater shortfalls in the numbers of Delta and Epsilon GSTs and CYP4 P450s, members of which clades have been recurrently associated with insecticide resistance in other species. It has been hypothesised that such shortfalls may contribute to the sensitivity of the honey bee to insecticides. On the other hand there are some recent radiations in CYP isoforms (CYP6, CYP9) and certain CCE clades in *A. mellifera* that could be associated with the evolution of the hormonal and chemosensory processes underpinning its highly organized eusociality (Johnson, 2008).

Regarding CYP genes, honey bees have one of the lowest number of isoforms of any inveterbrate sequenced to date (46 sequences), with the exception of fleas (*Pediculus humanus humanus*) (37 sequences) compared to 89 in *Drosophila melanogaster* and 111 in *Anopheles gambiae.* In comparison with other hymenoptera, the sequencing of the genome of the parasitic wasp (*Nasonia vitripennis),* which is haplodiploid as is A. *mellifera, has* revealed that this solitary parasitoid has twice as many CYPs as the honey bee with 92 CYP isoforms encoded in its genome. The difference between these two insects is most striking in the CYP4 clan, a poorly characterized group of CYP since *N. vitripennis* codes for 29 CYP4 P450s while *A. mellifera* includes only four. The CYP3 clan, which is associated with xenobiotic metabolism in other insects, is also reduced in *A. mellifera* compared to *N. vitripennis*. This pattern provides evidence that the well-regulated nest environment and diet of A *mellifera* constitute the principal factors in the low number of encoded CYP genes (Claudianos et al., 2006). From an evolutionary perspective, eusociality in bees and the high level of nest homeostasis insulate the queen from exposure to toxins making CYP-mediated detoxification less critical compared with other insects. Additionally, bees have a long evolutionary history of consuming processed nectar and bee bread resulting in a specialised exposure to phytochemicals and a low exposure to other environmental toxins, reducing the need for detoxicative enzymes (Claudianos et al., 2006; Johnson, 2008). These particular life style features of bees may explain the evolution of a lower number of CYP isoforms and the expression of specific CYP isoforms compared with other insects.

As an example, the CYP6AS subfamily (isoforms 1-10), which is apparently unique to hymenopterans is relatively dominant in honey bees. A number of CYP6AS isoforms (CYP6AS1, CYP6AS3, CYP6AS4) have been shown to play a role in processing phytochemicals encountered by bees in diet from concentrated processed nectar and bee bread. Indeed, quercetin, a compound present in honey, is a substrate for CYP6AS1, CYP6AS3 and CYP6AS4 isoforms and induces transcription of all three genes (Mao et al., 2011). Other isoforms have also been shown to be induced by honey extracts (CYP9Q2/CYP9Q3) suggesting that diet-derived phytochemicals may be natural substrates and may influence the ability of bees to detoxify pesticides (Johnson et al., 2009; Mao et al., 2011).

6.5.3.2 Cumulative and Synergistic effects of pesticides in honey bees

Prochloraz and deltamethrin‘s synergistic interactions were investigated in summer and winter bees. Individual compounds were used at sub-lethal doses that did not induce any significant mortality. Bees were treated with different doses of deltamethrin, either alone or in combination with prochloraz, at the constant field rate of 25 g/ha. In summer bees, the combination of prochloraz and deltamethrin at 125 mg/ha triggered a synergy that produced approx. 63 % mortality (corrected) after 24 h and at 62.5 mg/ha, deltamethrin’s synergy with prochloraz induced about 32.5 % mortality (corrected) after 24 h. and the field rate of 31.2 mg/ha was the lowest dose at which deltamethrin acted in synergy with prochloraz in summer bees. In winter bees, no synergy occurred between prochloraz and deltamethrin at doses of 125 and 250 mg/ha and synergy was only observed at a deltamethrin dose of 500 mg/ha and produced 48% mortality (corrected) after 24 h. Overall, summer bees were shown to be approximately eightfold more susceptible than winter bees to the synergistic action of prochloraz and deltamethrin (Meled et al., 1998).

Pilling et al. (1995) published in vivo studies on [14C]-l-cyhalothrin metabolism showing that prochloraz inhibits pyrethroid metabolism in honey bees. Thus, Meled et al (1998) concluded that higher CYP- metabolism in winter bees compared with summer bees would support the argument that prochloraz inhibits pyrethroid metabolism. Conversely, a lower oxidative metabolism of pyrethroids in winter bees than in summer bees would be consistent with the hypothesis that deltamethrin is distributed to the tissues more readily and is more toxic.

Thompson and Wilkins (2003) have assessed the synergy and repellency of combinations of pyrethroids /fungicide mixtures in bees using acute toxicity tests (LD_{50}) and consumption of sucrose respectively. Two pyrethroids (alpha-cypermethrin and lambda-cyhalothrin) and 8 fungicides (iprodione and thiophanate-methyl, carbendazim, prochloraz, chlorthalonil, flusilazole, difenconazole, propiconazole, tebuconazole) and their realistic combination were tested. Overall, six and three of the eight funcigides increased the toxicity of lambda-cyhalothrin and cypermethrin respectively, with a maximum decrease in LD_{50} and increase risk of 6.7 and 2.2 fold for lambda-cyhalothrin- prochloraz and alpha-cypermethrin –prochloraz.

Johnson et al. (2006) examined the effects of three compounds inhibiting CYP activities (piperonyl butoxide, PBO), carboxylesterases (COEs): S,S,Stributylphosphorotrithioate (DEF) and glutatione-s-transferases (GSTs) (diethyl maleate, DEM) on the toxicity of these pyrethroids cyfluthrin, lambda-cyhalothrin or tau-fluvalinate (table 1). Inhibition of P450s with PBO significantly enhanced the toxicity of all three pyrethroids tested, while inhibition of COEs with DEF significantly enhanced the toxicity of cyfluthrin and tau-fluvalinate. One hour after treatment with cyfluthrin, lambda-cyhalothrin or tau-fluvalinate, honey bees displayed ataxia or hyperactivity, depending on the dose, as expected for poisoning caused by pyrethroids. Across all treatments, toxicity of the three pyrethroids to adult bees varied by almost four orders of magnitude, with the greatest toxicity exhibited by cyhalothrin synergised with PBO (LD50 = 1.3 ng per bee) and with the lowest toxicity exhibited by tau-fluvalinate without inhibitor treatment (LD50 = 9450 ng per bee). The rank order of the toxicities of the three pyrethroids to honey bees was cyfluthrin (LD50 = 68 ng per bee) >cyhalothrin (LD50 - 103 ng per bee) »tau-fluvalinate (LD50 = 9450 ng per bee). In quantitative terms, toxicity of the three pyrethroids to bees was greatly synergised by the CYP inhibitor PBO whereas little synergism was observed for

the glutatione-s-transferases. Cyfluthrin which was relatively more toxic without enzyme inhibitors showed the least synergism (30-fold). Such results suggest that metabolic detoxification reactions, especially those mediated by CYP isoforms contribute significantly to honey bee tolerance to pyrethroid insecticides.

In terms of CYP inhibition, complex effects on the pyrethroid bifenthrin have been shown on CYP transcripts from honey bee mid-gut using RT-PCR with an induction of CYP9Q1 and CYP9Q2 and a repression of CYP9Q3 transcripts (Mao et al., 2011).

Laboratory bioassays were conducted to determine the contact honey bee toxicity of nitro and cyano substituted neonicotinoid insecticides applied to the dorsum of the honey bee thorax. Nitro-substituted compounds were the most toxic with LD50 values of 18 ng/bee for imidacloprid, 22 ng for clothianidin, 30 ng for thiamethoxam, 75 ng for dinotefuran and 138 ng for nitenpyram whereas cyano-substituted neonicotinoids exhibited a much lower toxicity with LD50 values for acetamiprid and thiacloprid of 7.1 and 14.6 mg/bee, respectively. CYP and COE inhibitors PFO and DEF, and DMI fungicides (triflumizole, propiconazole) were applied to anesthetized bees 1 h prior to the insecticide application at the level of 10 µg per bee. CYP and COE inhibitors and DMI fungicides increased honey bee toxicity of acetamiprid 6.0-, 244- and 105-fold and thiacloprid 154-, 1141- and 559-fold, respectively, but had a minimal effect on imidacloprid (1.70,1.85 and 1.52-fold,respectively) whereas GST inhibitors had no effects on toxicity compared with controls. In contrast, Acetamiprid metabolites, N-demethyl acetamiprid, 6-chloro-3-pyridylmethanol and 6-chloro-nicotinic acid when applied topically, produced no mortality at 50 mg/bee (Iwasa et al., 2004).

Recently, experiments were conducted at FERA (Thompson, personal communication) to investigate the dose dependency of the synergy between thiamethoxam and propiconazole in honey bees measured by changes in the LD_{50} of contact doses (estimated exposure level =0.224 µg/bee). The magnitude of the interaction at estimated exposure level was 1.5-fold (see Figure 6.2).

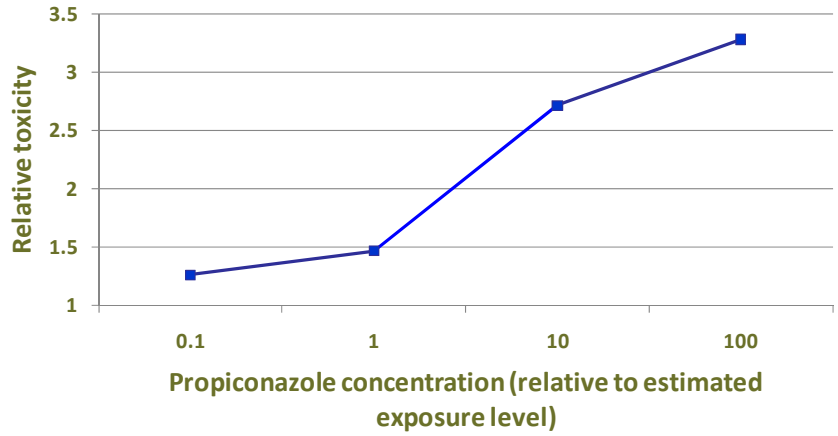


Figure 6.2: Increase in toxicity of thiamethoxam contact dose resulting from the co-application of propiconazole at differing doses (1= estimated exposure level = 0.224 µg/bee)

It is worth noting that in a number of papers on synergistic effects in bees, show synergists are several to 10 times above estimated environmental exposure in bees so that the magnitude of the interaction is an overestimation of the magnitude of the interaction that may be observed at an estimated level of exposure of synergists. Such data is not available for sub-lethal effects.

6.5.3.3 Synergistic effects between miticides applied to bee hives

Recently, Johnson et al. (2009) investigated the synergistic effects of the two miticides coumaphos and Tau-fluvinate by injecting the mixture in the thorax of three- to four-day-old bees using a microliter syringe mounted on a Hamilton PB-600 repeating dispenser. Pretreatment with CYP and COEs inhibitors exhibited a synergistic interaction with coumaphos, enhancing toxicity 2.8-fold and 4.0-fold, respectively whereas GST inhibitors did not have any influence. Synergism of tau-fluvalinate was also observed with coumaphos pre-treatment, with its toxicity exhibiting 2.1-fold synergism in the presence of as little as 0.3 µg coumaphos. Tau-fluvalinate toxicity increased in a dose-dependent manner with coumaphos as a pre-treatment increased: 2.5-, 4.4-, and 32.1-fold with 1 µg, 3 |µg, and 10 µg coumaphos respectively. The rationale for such interaction has been discussed previously and two important aspects would need to be considered: lipophilicity and competitive antagonism of the CYP enzyme. Both compounds are lipophilic and are absorbed by the wax component of the hive and can be persistent with the potential to build up over repeated treatments. Molecular modelling of the CYP9Q proteins and docking of tau-fluvalinate and coumaphos has recently shown that both miticide forms compete for the CYP isoforms resulting in competitive inhibition and decreased elimination of the compounds (Mao et al., 2011).

Recent data on P-glycoprotein inhibition and pesticide, miticide toxicity

P-glycoprotein or multiple drug resistance (MDR) transporters in bees have been recently shown to play a role in the synergistic effects of pesticides. Hawthorne and Dively (2011) showed that pre-treatment with a P-glycoprotein inhibitor (using the anti-hypertension drug verapamil) or the in-hive antibiotic (oxytetracycline) fed to bees significantly increased the toxicity (% mortality) of three neonicotinoid insecticides (imidacloprid, acetamiprid and thiacloprid insecticides) and two acaricides (coumaphos and t-fluvalinate). Increased mortality at higher concentrations and at the later end point (48 h) was observed for thiacloprid, and at 48 h for imidacloprid. For comparison with the verapamil synergism, mean bee mortality treated with 2 ug/ul coumaphos increased from 7% (n= 4 cages) to 51% (n= 4 cages) following feeding of OTC (1.4 mM), a significant but smaller increase than that caused by verapamil. OTC feeding increased the mortality of bees treated with 3 ug/ul t-fluvalinate from 5.6% (n= 10 cages) to 39% (n= 8 cages). The authors concluded in this preliminary study that all 5 compounds are substrates of one or more MDR transporters.

Overall, synergistic interactions in bees have been shown to result from toxicokinetic interactions at the level of metabolism either through the inhibition of a CYP or a transporter which then have toxicodynamic consequences enhancing the toxicity of the mixture/decreasing the LD50. However, full dose responses of such synergistic effects from potential inhibitors and different classes of pesticides are not available so that predictions of the magnitude of these interactions cannot be quantified in bees.

6.5.3.4 Synergistic effects between disease, malnutrition and pesticide toxicity in honey bees

Diseases and bee health

A number of bee diseases may result in adverse effects on bee health (Genersch et al., 2010), causing colony weakening or colony collapses. The most important diseases that occur regularly in Europe often spread from infestations with the *Varroa* mite, secondary infections with viruses (like Acute bee paralysis virus, Chronic bee paralysis virus, Deformed wing virus, Kashmir bee virus, Israeli acute paralysis virus) or infestations with the fungus *Nosema apis* or *Nosema ceranae* and other diseases such as European or American foulbrood. Such *Varroa* mites, *Nosema* spores and viruses are present in almost all colonies and may have severe impacts on colonies. Additionally, a number of factors influence the potential outbreak of clinical symptoms and are subject to great variation.

In semi-field or field trials with honey bee colonies, it is not possible to get comparable infestation rates with e.g. *Nosema* and *Varroa* or Viruses as it is in the laboratory. Indeed, *varroa* has not yet been

grown *in vitro* and achieving a standardised infection rate of bees and brood is difficult. Often, virus loads are detected in colonies without any clinical symptoms and factors leading to clinical symptoms are not yet fully understood. Infection with *Nosema* spores are difficult to handle and a controlled infection is only possible in the laboratory but still with some difficulties. Variability in response to *Nosema* in bees is very large. For example, during a *Nosema apis* infection in *Apis mellifera* with several different spore doses, it was not possible to establish a discernible relationship between bee longevity and spore dose in two races of European bee (Malone and Stefanovic, 1999). Infections of *N. ceranae* appear to have different effects on colonies in different geographical regions. Finally, seasonal variations and gross colony level symptoms (Fries, 2010) described for *N. apis* do not seem to be present in *N. ceranae*.

Recently, gene expression profiles from whole-genome microarrays between guts of bees from Colony Collapse Disorder (CCD) colonies originating on both the east and west coasts of the United States and guts of bees from healthy colonies sampled before the emergence of CCD, have been published. Indeed, the gut of bees acts as a primary interface between the honey bee and its environment (as a site of entry for pathogens and toxins). First, considerable variation in gene expression was associated with the geographical origin of the bees, although 65 transcripts were identified as potential markers for CCD status. Overall, no elevated expression of pesticide response genes was observed and genes involved in immune response showed no clear trend in expression pattern despite the increased prevalence of viruses and other pathogens in CCD colonies. However, unusual ribosomal RNA fragments, depicted through microarray analysis, were more abundant in the guts of CCD bees. The authors concluded that such fragments may be related to arrested translation as a possible consequence of picorna-like viral infection (including Deformed wing virus and Israeli acute paralysis virus) and that analysis of the RNA fragments' abundance and presence maybe a useful diagnostic marker for the CCD bee (Johnson et al., 2009).

Synergistic effects between diseases and pesticide toxicity in honey bees

There is growing evidence that interaction between honey bee disease and pesticide toxicity have synergistic effects on bee health. Alaux et al. (2010) investigated the interaction between the microsporidia *Nosema* and imidacloprid (0.7, 7 and 70 µg/kg) and showed increased sucrose consumption (i.e. increase in imidacloprid exposure) and consequently increased mortality. Overall, the combination of both agents caused, in the short-term, the highest individual mortality rates compared with controls. Haemocyte number and phenoloxidase activity, as markers of immunity, were quantified for both individual and social levels but neither markers were shown to be affected by treatment. In contrast, glucose oxidase activity used in honey bees for the sterilisation of the colony and brood food, was significantly decreased suggesting a synergistic interaction and in the long-term a potentially higher susceptibility of the colony to pathogens. Vidau et al (2011) showed synergistic effects on bee mortality between exposure to sub-lethal doses of fipronil or thiacloprid and *N. ceranae* infection compared with uninfected bees. Induction of phase I and phase II detoxification enzymes were also measured in mid-gut of the bees and only phase II (glutathione-s-transferases) were shown to be induced whereas phase I (cytochrome P-450 activity measured as 7-ethoxycoumarin-O-deethylase) was not. The authors also tested the effect of insecticide exposure on *Nosema* spore production. Fipronil and thiacloprid were shown to have opposite effects on spore production with a respective decrease and increase of about 33% and 40 %. The authors concluded that these results did not explain the mortality increase observed in the presence of insecticides and that further research is needed.

Recently, the infection dynamics of deformed wing virus (DWV), sacbrood virus (SBV), and black queen cell virus (BQCV) in adult bees, *Varroa* mite-infested pupae, s, and uninfested pupae, has been compared between bees treated with tau-fluvalinate and untreated control colonies. Initially, titres of DWV increased with the onset of the acaricide application and then slightly decreased progressively coinciding with the removal of the *Varroa* mite infestation and the authors concluded that the initial increase in DWV titres suggested a physiological effect of tau-fluvalinate on the host's susceptibility to viral infection. DWV titres in adult bees and uninfested pupae remained higher in treated colonies

than in untreated colonies. The titres of SBV and BQCV found had a variety of possible effects of tau-fluvinate (Locke et al., 2012).

One of the challenges raised by these studies is the difficulty to extrapolate to field conditions since comparable field studies have never been published and comparable infections under field conditions are very difficult, if not impossible, to achieve.

Malnutrition

The shortage of food may result in adverse effects on bee colonies. A lack of carbohydrates may result in weakening and consequently death of a bee colony whereas a lack of pollen will result in brood reduction, brood cannibalism, resulting in colony weakening and poor health status. Hence, good beekeeping practice would ensure that bees have access to good nutritional sources and that the landscape offers good foraging throughout the year. In normal conditions, also in agricultural areas, beekeepers are able to and will choose an appropriate location ensuring nectar and pollen flow. Beekeepers may feed sugar syrup to avoid carbohydrate starvation according to good beekeeping practice.

Bees fed high quality pollen appear less sensitive to pesticides than those fed with lower qualities or inadequate amounts of pollen or pollen substitute during development (Wahl and Ulm, 1983, von der Ohe and Janke, 2009). The amount of pollen collected by a colony could potentially be influenced by as many as 10 or more other variables (e.g. worker population size, number of larvae, surrounding vegetation, weather conditions etc.) (Keller et al., 2005). Nevertheless, the deprivation of protein status cannot be standardised for bee colonies. It is important to point out that policy makers should ensure that planting flowering crops or flower strips, maintaining and promoting biodiversity is of major importance to the health of honey bees and non-*Apis* bees and especially for maintaining non-*Apis* populations.

A recent study demonstrated, through an analysis of gene expression in bee midguts using northern blots, that honey, pollen and propolis induces detoxification enzymes in bees (CYP6AS), through the natural flavonoid quercetin and that mortality in bees exposed to the mycotoxin aflatoxin consuming sucrose or high-fructose corn was higher compared with bees exposed to aflatoxin and fed honey (Johnson et al., 2012).

Overall, there is a growing body of evidence that bees infected by parasites/pathogens may be more susceptible to chemical toxicity than healthy ones and that malnutrition also influences bee health. Active monitoring may be designed as “multifactorial studies” to investigate multiple factors putatively contributing to bee mortality including diseases. There is a wide variety of monitoring and surveillance systems for bee mortality and bee health which have been recently reviewed by an EFSA working group (Hendrikx et al., 2009).

6.6 Cumulative and synergistic effects

6.6.1 How to calculate concentration and response addition

Concentration addition (CA)

This approach is used where chemicals have the same site of action (simple similar joint action) but do not affect the biological activity of each other (no interaction). For this method the endpoint must be the same for each chemical.

$$\text{Total Toxicity} = (C_a/T_a + (C_b/T_b) + \ldots\ldots + (C_n/T_n) \qquad \text{Concentration addition (CA)}$$

Where C = concentration (or dose)
T = toxicity

Response addition (RA)

This approach is used where chemicals have different sites of action (independent joint action) but do not affect the biological activity of each other (no interaction). Here each component of the mixture acts on a different physiological or biological system but contributes to a common response.

This requires biological response (BR) expressed as % toxic effect for the assessed concentration from dose response curve for each constituent.

Total toxicity = $BR_1 + BR_2 + ... + BR_n$ Response addition (RA)

The disadvantage of this method is that it requires dose response data for all of the mixture constituents and species being assessed.

6.6.2 Comparisons of additive estimates with measured toxicity

There is evidence that CA is a conservative method for assessing the toxicity of mixtures (Kortenkamp et al., 2009; Verbruggen and van den Brink, 2010). In all cases analysed, the estimated toxicity using this approach was higher than that predicted by IA (Kortenkamp et al., 2009). When comparing estimates using CA, it has been estimated that the majority of estimates do not deviate by more than a factor of 2 (Deneer, 2000), 2.5 (Warne, 2003; Junghans et al., 2006) or 3 (Kortenkamp et al., 2009). There is also some evidence that this deviation is greatest for mixtures containing small numbers of chemicals and decreases as the complexity of the mixture increases. With respect to honey bees, the analyses performed by Deneer (2000), Warne (2003), Junghans et al. (2006), Kortenkamp et al. (2009) did not include toxicity data for honey bees. In addition, the mixture toxicity data presented in section 6.5 shows that in some cases magnitude of interactions can be higher than a factor of 3 although most studies have used synergist concentration which were often orders of magnitude above environmental concentrations. Finally, because of their specific toxicokinetic profile compared to other insects, synergistic interactions between pesticides in bees have a toxicokinetic basis (see section 6.5) and full dose responses of synergistic effects from potential inhibitors and different classes of pesticides at concentrations of environmental relevance.

Hence, applying a default uncertainty factor, such as an uncertainty factor of 2 to 3 to the threshold of toxicity for honey bees, would be premature until laboratory research has been undertaken with a number of mixtures of priority at relevant levels of exposure in adult bees and larvae.

6.6.3 Proposal for assessment of cumulative and/or synergistic effects

Synergism of pesticides in honey bees can either be predicted or assumed based on chemical class information (e.g. conazole (EBI) fungicides and insecticides) and knowledge of the mode of action/molecular targets of the individual pesticides in the mixtures. Therefore, in the absence of existing data on toxicity of the mixture, it may be necessary 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. Finally, if the compound has the potential for bioaccumulation and repeat dose effects (Chapter 4), the risk assessment scheme proposed in Chapter 6 would require data for half life of the compound and its metabolites in adult bees and larvae. Such toxicokinetic information can provide a further understanding of the likelihood of pesticide synergism in honey bees.

The design of ecotoxicological studies for mixtures of potential synergists should take into account the toxicokinetics of the synergists (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 concentrations for which no potentiating factor may occur in the dose response curve.

Such statistically-sound dose response data will provide a basis to derive benchmark doses (BMD) and their limit (BMDL) as suggested by EFSA's Scientific Committee (EFSA, 2009b). Such benchmark doses are very rarely available in ecotoxicology and provide a flexible approach to reaching quantitative protection goals. For example, the dose response can consider a specific effect size for lethality (1%, 2% or 5%) or for a sub-lethal effect (described in Chapter 4-annexes) depending on the protection goal and the aim of the risk assessment. In this case, such a BMDL would be equivalent to an SSD (percentile of species affected by the exposure to multiple substances) (Posthuma et al., 2002) which is already applicable to concentration addition and response addition and can be applied to synergism.

Such a mechanistic approach has been proposed by the three non-food committees of the European Commission when dealing with ecotoxicological data of mixtures while acknowledging that very little mechanistic data is available in this field (SCHER/SCENIHR/SCCS, 2011). 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 assessments i.e. to derive species-specific, chemical-specific, mixture-specific or class- specific adjustment factors using the full dose responses, the BMDs, BMDLs or SSDs and the magnitude of the interaction.

7 CHAPTER 7: RISK ASSESSMENT FOR HONEY BEES, BUMBLE BEES AND SOLITARY BEES

7.1 Summary

In Chapter 7 two new risk assessment schemes are proposed: one for honey bees and one for bumble bees and solitary bees[9]. The three most important exposure routes are included. In former chapters it was highlighted that the most important exposure routes are: exposure of foragers by sprayed products or dust (either by contact and/or oral intake), exposure of in-hive bees (including the larval stages) by compounds brought in by pollen and/or by nectar, and exposure to compounds that can be translocated in the plant to pollen, nectar or guttation droplets (for instance systemic compounds).

In the first tier it is proposed to include toxicity testing that covers a longer period of exposure (7 to 10 days) for adult bees as well as larval bees. Both life stages can be exposed for more than one day and this risk is not covered by the standard OECD tests (213 and 214) for oral and contact exposure. Currently there is insufficient evidence that toxicity following extended exposures can be reliably predicted from acute oral LD50 data. It is also proposed to investigate for each compound whether there are any indications of cumulative effects.

If the LD50 for the adult bee is greater than 100 µg/bee and there is no evidence of cumulative toxicity in larvae in the 7 day larval test (Aupinel) then no further larval tests are required and the NOEL (chronic 7 day larva) is used in risk assessment. In all other cases an Oomen et al (1992) type study is required to integrate brood care behaviour of the adult bees and the lower NOEL from the Aupinel and Oomen is used in the scheme.

The scheme for bumble bees and solitary bees is based on less well defined steps and toxicity tests. The scheme uses data for honey bees as surrogate input. The development of standardized contact and oral toxicity test, semi field and field tests for bumble bees and solitary bees has yet to be completed and ring tested. There is a need to improve the testing protocols concerning bumble bees and solitary bees, in particular to better address the chronic risk and the identification and measurement of sub-lethal effects (e.g. effects on memory, learning capacity, orientation) to be used in the risk assessment. The primary concerns for bumble bee and solitary bee species were considered to be from insecticides, pesticides with insecticidal activity and insect growth regulators and therefore the risk assessment proposed is primarily for these modes of action. However, the honey bee scheme has to be applied for each compound unless the risk can be characterized as negligible. Note that when the exposure for bees is classified as negligible, the exposure for bumbles bees and solitary bees could still be possible.

7.2 Introduction

In former chapters it has been highlighted that the most important exposure routes are:

- exposure of foragers by sprayed products or dust (either by contact and/or oral intake),
- exposure of bees (including the larval stages) by compounds in pollen and/or in nectar as a result of exposure of spray or dust and in pollen, nectar or guttation droplets as a result of systemic translocation in plants.

For these routes, as well as for bees as for bumble bees and solitary bees, a proposal for a new risk assessment scheme is provided.

[9] If necessary amendments will be made to the risk assessment schemes in the follow up project of this opinion: the preparation of a guidance document for honey bees, bumble bees and solitary bees

It is anticipated that the studies which are proposed in the risk assessment schemes below are the studies with the improvements recommended in Chapter 5.

Acute and chronic risk assessment

For risk assessment of adult honey bees following a spray application, the contact and oral acute (single dose) LD50 should be generated (using OECD guidelines 213 and 214) as these reflect the hazard associated with single acute exposures. Both routes of exposure should be evaluated as there is currently insufficient data to predict the contact LD50 from the oral LD50 and vice versa. It is important that the OECD guidelines are complied with in detail, e.g. that the study is extended if increasing mortality is observed and all sub-lethal effects are reported. Data on the toxicity of the active ingredient and the formulation should be reported (LD50, ECx and slope) as effects may differ, e.g. co-formulants may alter the rate of uptake and products may contain more than one active ingredient. These data are used to generate the Hazard Quotient (HQ) using the lowest of the LD50 estimates and the application rate (µg ai or µg product as appropriate) at the first tier. Although the HQ is not based on a detailed assessment of exposure to sprayed products it is a measure of risk which has been validated using field trial and incident data (Thompson and Thorbahn, 2009).
For systemic pesticides applied as seed and soil treatments exposure may be by intake of contaminated nectar, through guttation water or via dusts. As for the sprayed compounds the acute oral LD50 should be evaluated but the contact exposure route is less relevant.

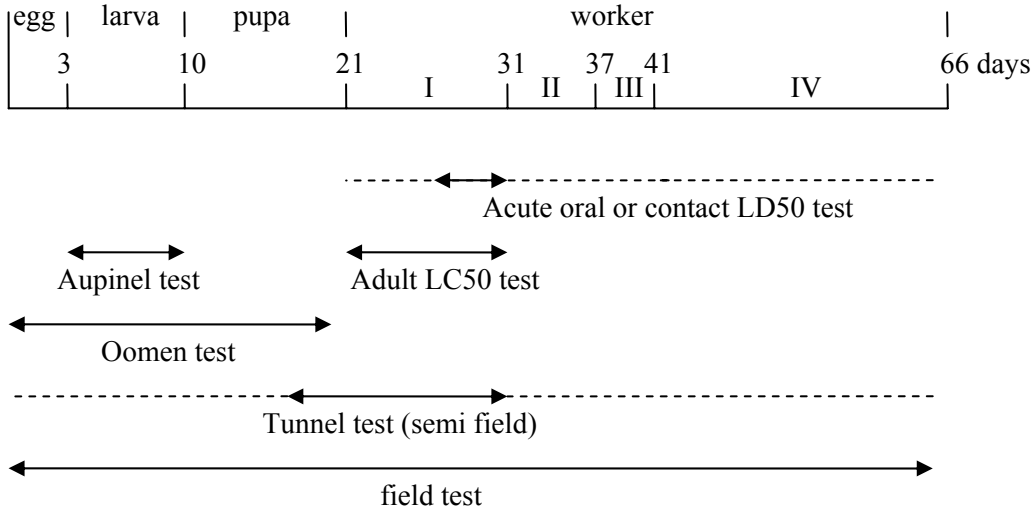
It is recognised that single acute exposure scenarios are not representative of the exposure of foragers or in-hive honey bees for compounds which may persist for more than a single day in the environment, or in nectar and/or pollen returned to the hive. Currently there is insufficient evidence that toxicity following extended exposures can be reliably predicted from acute oral LD50 data. Until this can be demonstrated, a more extended oral toxicity study is recommended; in practice even when the database supports prediction for existing classes of active ingredient it is recommended that these are conducted for active ingredients for new classes of active ingredient. Oral extended exposure studies should be undertaken for both the active ingredient and the product (detailed harmonised guidelines for their conduct are required) and again any observed sub-lethal effects should be reported. The data should be used to determine both the LC50 and NOEC and ECx and to investigate whether there are any indications of cumulative effects according to Chapter 4. Currently there is no data to support an HQ approach and therefore a more standard ETR approach is recommended based on the exposure of the adult honey bees and the LC50, NOEC and ECx.

Insect growth regulators are a specific class of insecticides known to affect brood and not adult honey bees. Therefore all active ingredients and formulations with IGR properties must be assessed using the Oomen et al (1992) brood dosing study to generate a NOEC as this covers all stages until emergence. Although Oomen et al (1992) is not recognised as a fully validated guideline the test methodology has been used for a number of years and there is extensive experience in its conduct and interpretation. It is recommended that it is submitted for consideration as an international guideline.
For compounds within the hive acute exposure of larvae is unlikely to occur and a chronic exposure is a more realistic scenario. At present there are insufficient data available to predict the toxicity to larvae from that in adults. Therefore until data is available to support such predictions chronic toxicity studies (exposure for the developmental period of the larvae as a minimum) should be conducted with both the active ingredient and the product (for spray applications) to ensure the safety of co-formulants returned to the hive on pollen and in nectar after spray applications are assessed. These studies may be conducted with a laboratory study (similar to that proposed by Aupinel et al. (2009) but to cover the chronic dosing scenario) or by adaptation of the Oomen et al. (1992) study to generate dose-response data. Neither of these test methods are currently recognised as validated guidelines and it is recommended that this is considered as a priority. The data should be used to both determine the NOEC and ECx and to investigate whether there are any indications of cumulative effects according to Chapter 4 (for bee-toxic compounds it is more appropriate to use a laboratory study where daily

assessments are possible). Again a more standard ETR approach is appropriate based on the exposure of the larvae and the NOEC or ECx.

In Figure 7.1 the parts of a bee life cycle covered by the toxicity tests are depicted. The acute oral or contact test only covers a small part of the honey bee worker stage (preferably bees from the cleaning and feeding phase of the worker bee life cycle). The Aupinel test covers the larval stage and the Oomen tests the egg, larval and pupal stage through to emergence. The semi-field exposure phase within the tunnel is limited to 10 -14 days as this is as long as a colony can be kept within a tunnel without adverse effects on development, but they can be moved outside and kept for as long as is required. A field test can be kept as long as required, for instance when the hive is kept for 63 days in the field it will cover 3 brood cycles.

Figure 7.1: Part of the bee life cycle (i.e. worker bee) potentially covered by toxicity tests



I = cleaning and feeding phase, II = wax producing and cell construction phase, III guiding and ventilating phase, and IV forager phase

Trigger values

Trigger values have not been included in the discussion of the proposed risk assessment approach as these should be established and calibrated as part of the development of the guidance document.

Semi-field studies

Well-designed semi-field studies are considered as the worst-case exposure scenario (equivalent to at least 95% exposure scenario) as honey bees are confined to the treated crop. Due consideration should be given to the design of the semi-field studies to ensure that the crop is highly attractive (e.g. Phacelia) and that colonies are exposed to the treated crop, e.g. spray applications during periods of active foraging, removal of stores prior to exposure. For systemic compounds it is recognised that the exposure may be limited in semi-field studies due to the area of forage available. Therefore it is recommended that consideration be given to improvements to the OECD75 test design for systemic pesticides to extend the exposure period, e.g. by providing supplementary pollen and sucrose sources which contain the same residue levels as the treated crop and extension of the study to encompass a suitable post-exposure assessment period depending on the persistence of the chemical. The conduct

of the semi-field studies should always take into account the findings in previous studies, e.g. if the study is triggered by concerns about adult acute mortality and sub-lethal effects then these aspects should be studied in detail in an EPPO 170 test design, e.g. behaviour of foragers, behaviour at the hive entrance, if the study is triggered by the larval study then a OECD 75 study design is appropriate. If concerns are raised by effects on both adults and larvae then further adaptation of OECD 75 is required to address adult effects identified in EPPO 170, e.g. behaviour of foragers, behaviour at the hive entrance and daily mortality in addition to detailed assessments of brood.

Field studies

Field studies are considered as realistic but not worst case when compared to semi-field studies and if well-designed may be identified as realistic worst case (i.e. the x^{th} percentile). However, to achieve this due consideration should be given to ensuring that exposure is maximised in the study, e.g. the use of a highly attractive crop and minimisation of alternative forage sources around the treated area, removal of stores prior to exposure and extension of the assessment period to ensure effects can be detected. As for semi-field studies the endpoints should be directed primarily to the concerns raised by the previous studies but also encompass sub-lethal effects, e.g. on foraging activity.

Details regarding methodology for assessment of uncertainties have not been included in discussion of the proposed risk assessment approach as these should be established as part of the development of the guidance document.
Risk management has not been included in discussion of the proposed risk assessment approach as these should also be established as part of the development of the guidance document.

Exposure assessment in the risk assessment scheme

The risk assessment schemes for honey bees, bumble bees and solitary bees require exposure concentrations in order to calculate the ETR quotients at a number of places. As described in Chapter 2, the aim of the exposure assessment is to consider a x^{th} percentile case. So all the exposure concentrations in these risk assessment schemes should be equal or higher than a x^{th} percentile case. These risk assessment schemes contain semi-field or field studies in the higher tiers at a number of places. These studies usually only consider one treatment level that is compared to an untreated control. To be consistent with the exposure assessment aim, the exposure in these semi-field or field studies should be equal to or higher than a x^{th} percentile case.

Risk assessment for bumble bees and solitary bees

The primary concerns for **bumble bees and solitary bees** were considered to be from insecticides, insecticidal and IGR pesticides and therefore the risk assessment proposed is primarily for these modes of action. A lower trigger should be used in the first tier of the bumble bee and solitary bee risk assessment than that used in the honey bee risk assessment to take account of the cross-species extrapolation following acute and chronic exposure. Additional exposure scenarios highlighted in Chapter 3 may be important for **bumble bees and solitary bees**, e.g. soil, and further research is needed to determine their relative importance and, if required, inclusion in risk assessment.

There is a need for research to develop relevant standardised semi-field and field test designs for bumble bees and solitary bees. In some cases, e.g. bumble bees, these may be relatively straightforward, but for other species, such as univoltine solitary bees, methodology requires significant further work.

7.3 Risk assessment scheme for honey bees

1 Collect details of the product and its pattern of use.
E.g. type of application (sprayed products, granular products, seed treatment, etc.), time of treatment in relation to crop flowering and any special direction for use (including mitigation),

attractiveness of crop to bees, whether the active ingredient or metabolite is systemic and how the application fits within crop rotation, etc.

go to 2

2 Is exposure for bees negligible (see Note 1)?

if yes classify risk as negligible
if no go to 3

3 How are the bees exposed?

If active ingredient is applied as a spray go to 4
If active ingredient is applied as a granule or as seed treatment go to 11
If active ingredient does not fit in any of the above questions go to 14

Remark: it is possible that a certain compound ticks more than one box. In that case all applicable routes have to be assessed

Exposure to sprayed products

4 Assess whether the active ingredient can be systemic translocated in the plant to the pollen or the nectar or to the guttation droplets

If yes go to 5 <u>and</u> 6
If no go to 5

5 Assess the toxicity of the product to worker honey bees by conducting contact and oral laboratory studies (LD50) and oral LC50 over 10 days (see Note 2).

Calculate the Hazard Quotient (HQ, see Note 3) between the application rate and the lower of the LD50 toxicity values (g ha^{-1} ⁄LD50 in µg per bee).

Calculate the Exposure Toxicity Ratio (ETR_{adult}) between the amount of residues (see Note 4) that may be ingested by an adult bee in 1 day and the LC50 value.

Assess the toxicity (see Note 5) of the product to honey bee larvae with a chronic 7-day larval test (e.g. Aupinel) and a bee brood feeding test if relevant (e.g. Oomen et al., 1992).

Remark: If the LD50 for the adult bee is greater than 100 µg/bee and there is no evidence of cumulative toxicity in larvae in the chronic larval (Aupinel) then no further larval tests are required and the NOEL (chronic 7 day larva) below is used.
In all other cases (including IGR) an Oomen et al (1992) type study is required to integrate brood care behaviour of the adult bees and the lower NOEL from the Aupinel and Oomen is used in the scheme.

Calculate the ETR_{larvae} between the amount of residues that may be ingested by a larva in 1 day and the no observed effect level (NOEL).

Assess whether there is evidence of cumulative toxicity according to Haber's Law in the toxicity tests with adult and larval honey bees (see note 6).

if HQ < 50 and ETR_{adult} < X and if ETR_{larvae} < Y and no evidence for cumulative toxicity go to 10
if HQ ≥ 50 or ETR_{adult} ≥ X or ETR_{larvae} ≥ Y or evidence of cumulative toxicity go to 7 (see note 7)

6 Take the results of the oral LD50 and LC50 toxicity tests and the NOEL of the oral toxicity test with larvae.

Calculate the ETR_{acute} between the amount of residues that may be ingested by a bee in 1 day via guttation fluid, contaminated pollen and/or contaminated nectar (Daily intake per bee (µg) ⁄LD50 in µg per bee),

Calculate the ETR_{adult} between the amount of residues that may be ingested by a bee in 1 day via guttation fluid, contaminated pollen and/or contaminated nectar (Daily intake per bee (µg) ⁄LC50 in µg per bee),

Calculate the ETR_{larvae} between the amount of residues that may be ingested by a larva in 1 day and the no observed effect level (NOEL).

if HQ < 50 and ETR_{adult} < W and if ETR_{larvae} < X go to 10
if HQ ≥ 50 or ETR_{adult} ≥ W or ETR_{larvae} ≥ X go to 7 (see note 7)

7 Assess the effects of the product in semi-field tests (see note 8).
Do results indicate minimal risk (no significant difference to control)?
if yes go to 10
if no go to 8

8 Assess the effects of the product in field tests (see note 9).
Do results indicate minimal risk (no significant difference to control)?
if yes go to 10
if no go to 9

9 Analyze uncertainties to confirm the conclusions. Consider whether specific risk management measures are possible
if yes reassess the potential risk
if no classify risk as high

10 Classify as **low risk** for bees after analyzing the uncertainties.

Exposure via granular treatment and seed treatment

11 Assess whether the active ingredient can be systemic translocated in the plant to the pollen or the nectar or to the guttation droplets.
Assess whether honey bees can be exposed to dust during application
If the answer is yes to both questions go to 12 <u>and</u> 13
If exposure is only by dust go to 12
If compound is only systemic go to 13
If the answer is no to both questions go to 10

12 Assess the toxicity of the product to worker honey bees by conducting contact and oral laboratory studies (LD50) and oral LC50 over 10 days.

Calculate the HQ between the exposure (see Note 10) by dust (X^{th} percentile concentration (see note 11) and the lower of the LD50 toxicity values (g ha^{-1} /LD50 in µg per bee).
Calculate the ETR_{adult} between the amount of residues that may be ingested by an adult bee in 1 day and the LC50 value.

Assess the toxicity of the product to honey bee larvae with a chronic 7 day larval test (e.g. Aupinel) and a bee brood feeding test if relevant (e.g. Oomen et al., 1992).

Remark: If the LD50 for the adult bee is greater than 100 µg/bee and there is no evidence of cumulative toxicity in larvae in the chronic larval (Aupinel) then no further larval tests are required and the NOEL (chronic 7 day larva) below is used.
In all other cases (including IGR) an Oomen et al (1992) type study is required to integrate brood care behaviour of the adult bees and the lower NOEL from the Aupinel and Oomen is used in the scheme.

Calculate the ETR_{larvae} between the amount of residues that may be ingested by a larva in 1 day and the no observed effect level (NOEL).

Assess whether there is evidence for cumulative toxicity according to Haber's Law in the toxicity tests with adult and larval honey bees.

if HQ < 50 and ETR_{adult} < X and if ETR_{larvae} < Y and no evidence of cumulative toxicity go to 10
if HQ ≥ 50 or ETR_{adult} ≥ X or ETR_{larvae} ≥ Y and evidence of cumulative toxicity go to 7

13 Assess the toxicity of the product to worker honey bees by conducting an oral LD50 and oral LC50.

Calculate the ETR_{acute} between the amount of residues that may be ingested by a bee in 1 day via guttation fluid, contaminated pollen and/or contaminated nectar (Daily intake per bee (µg) /LD50 in µg per bee),
Calculate the ETR_{adult} between the amount of residues that may be ingested by a bee in 1 day via guttation fluid, contaminated pollen and/or contaminated nectar (Daily intake per bee (µg) /LC50 in µg per bee),

Assess the toxicity of the product to honey bee larvae with a chronic 7 day larval test (e.g. Aupinel) and a bee brood feeding test if relevant (e.g. Oomen et al., 1992).

Remark: If the LD50 for the adult bee is greater than 100 µg/bee and there is no evidence of cumulative toxicity in larvae in the chronic larval (Aupinel) then no further larval tests are required and the NOEL (chronic 7 day larva) below is used .
In all other cases (including IGR) an Oomen et al (1992) type study is required to integrate brood care behaviour of the adult bees and the lower NOEL from the Aupinel and Oomen is used in the scheme.

Calculate the ETR_{larvae} between the amount of residues that may be ingested by a larva in 1 day and the no observed effect level (NOEL).

Assess whether there is evidence for cumulative toxicity according to Haber's Law in the toxicity tests with adult and larval honey bees.

if ETR_{acute} < 10 and ETR_{adult} < X and if ETR_{larvae} < Y and no evidence of cumulative toxicity go to 10
if ETR_{acute} ≥ 10 or ETR_{adult} ≥ X or ETR_{larvae} ≥ Y or evidence of cumulative toxicity go to 7[10]

Other compounds not covered in the steps before

14 For novel application methods, a specific risk assessment method may need to be developed (e.g termite baits). As a minimum, reports of acute oral and contact tests and a chronic toxicity test shall be submitted.

Notes

Note 1 According to the latest version of the draft on regulatory requirements for active substances (SANCO, 2011) for seed treatments there needs to be a consideration of the risk from drift or dust during time of drilling the treated seed and for granules and slug pellets there needs to be a consideration of the risk from drift of dust during application (exposure of bees visiting neighbouring plants). If an active substance is systemic and to be used on seeds, bulbs, roots, applied directly to soil (e.g. sprayed on to soil, granules/pellets applied to soil, irrigation water) or directly into the plant (e.g. stem injection), then the risk to bees foraging those plants should be assessed, including the risk deriving from pesticide residues in nectar, pollen and water, including guttation.
Examples when exposure of bees is negligible: food storage in enclosed spaces, non-systemic preparations for application to soil, except granules, non-systemic dipping treatments for transplanted crops and bulbs, wound sealing and healing treatments, non systemic rodenticidal baits, and use in glasshouses without honey bees as pollinators.

[10] When there is evidence of cumulative toxicity the area available for foraging in tunnel studies is probably not large enough. Maybe in these cases the field area has to be increased to 10 ha for systemics to ensure they have nowhere else to forage and there is more than enough forage to ensure long term exposure.

Note 2 According to the latest version of the draft on regulatory requirements for active substances (SANCO, 2011) reports of acute oral and contact tests and a chronic toxicity test shall be submitted.
There is a need to improve the testing protocols concerning bees, in particular to better address the chronic risk to bees and the identification and measurement of sub-lethal effects (e.g. effects on memory, learning capacity, orientation) to be used in the risk assessment. Pending the validation and adoption of new test protocols and of a new risk assessment scheme, all efforts shall be put in place to comprehensively address, with the existing protocols, the acute and chronic risk to bees, including those on colony survival and development.
The tests shall provide the EC_{10}, EC_{20}, EC_{50} (or an explanation if they cannot be estimated) together with the NOEC. Sub-lethal effects, if observed, shall be reported.

Note 3 For sprayed products the basis of the acute risk assessment is the generation of a hazard quotient (HQ) calculated by dividing the application rate of the sprayed product's active ingredient by the contact or oral LD50 (whichever is the lower). In the past post-registration monitoring data collected in the United Kingdom in their Wildlife Incident Investigation Scheme have been used to calibrate the HQ. The HQ was set as 50. For values smaller than 50 low risk is predicted for bees. Recently this value was revaluated with incidents reported post-registration monitoring schemes of the UK, Germany and the Netherlands (Thompson and Thorbahn, 2009). This review of incidents in Europe over the last 25 years suggests that the HQ approach to risk assessment for honey bees offers an appropriate level of protection.
The risk from short-term exposure (approximately 10 days) to contaminated food ad libitum is based on an exposure toxicity scenario. Preliminary thoughts for trigger values are 1 when the toxicity endpoint is the NOEC and 10 when the endpoint is a LC50 value. A definitive proposal for the values X and Y in the honey bee scheme will be established as part of the development of the guidance document (see also Appendix R).

Note 4 Residue level in honey and pollen. According to the latest version of the draft on regulatory requirements for active substances (SANCO, 2011) residue levels in honey and pollen should be reported. Residue studies are normally required: when a plant protection product is used during or shortly before blossom of the crop or when a plant protection product is used before blossom and the active substance used has a low degradation rate and/or is systemic and when these flowering crops are used to produce pure blossom honey.
For environmental risk assessment, tests should preferably be conducted in semi-field or under worst case field conditions, using the plant protection product in accordance with the critical GAP. During peak flowering honey and pollen should be collected for analysis. Results from at least three trials performed at different geographical locations should be provided. To be consistent with the exposure assessment aim, the exposure estimate should be equal to or higher than a x^{th} percentile case.

Note 5 The bee brood test should provide sufficient information to evaluate possible risks from the active substance on honey bee larvae. The test shall be carried out for substances for which effects on growth or development cannot be excluded, unless it can be justified that it is not possible that honey bee brood would be exposed to the active substance, e.g. from the use of non-systemic seed treatments, non-systemic soil applied treatments, pre-flowering non-systemic sprays.

Note 6 Guidance for calculating whether an active ingredient shows evidence for cumulative toxicity according to Haber's Law can be found in Chapter 4.

Note 7 A semi-field test is needed where there is evidence of cumulative toxicity to demonstrate what happens in a semi-field environment where the honey bees have no option but to feed on the treated crop, i.e. whether there is concern at realistic exposure levels. For the time being it is proposed to require this study for all sprays to see if there are behavioural effects under worst case exposure until laboratory tests for sub-lethal effects are available and to provide the information to describe worst-case exposure.

Note 8 When laboratory studies trigger higher tier tests due to concerns on adult bees only, a semi-field according to the EPPO guideline 170 is the most appropriate test to carry out. If concerns are only triggered by the bee larvae test (either Aupinel or Oomen) a semi-field test according to OECD guideline 75 is the most appropriate test to carry out. If concerns for both adults and larvae are raised EPPO 170 and OECD 75 are appropriate to carry out (perhaps in a combined test design) i.e. daily mortality assessments for 28 days and special brood assessments. Probably a single semi field test will

not give the answer to whether the compound is safe to use in agriculture. A definitive proposal for the number of studies to be carried out and how severe the test circumstances have to be, will be established as part of the development of the guidance document for honey bees.

Note 9 Probably a single field test will not give the answer to whether the compound is safe to use in agriculture. A field study should have sufficient power to detect the effects above the suggested thresholds for the magnitude of effects. See also Chapter 2 (paragraph 2.2.3 and Figure 2.1) on the number of honey bee hives needed to detect specified increases in mortality rate (percentage effect size) using dead bee traps. A definitive proposal for the number of studies to be carried out and how severe the test circumstances have to be, will be established as part of the development of the guidance document for honey bees.

Note 10 It is believed that in case of equal amounts of exposure, that exposure of the compound solved in either water or a solvent could be considered more worst case than exposure of the compound in a dry phase (e.g. dust). There is some circumstantial evidence that this assumption could be true. In studies with normal humidity and high humidity, honey bee mortality after dust exposure was greater under high humidity circumstances (Mazaro et al., 2011). Data submitted for the peer review of the pesticide risk assessment of the active substance thiamethoxam shows that the acute toxicity data for oral exposure as well as contact exposure are comparable for a liquid formulation and a dust formulation (in liquid phase the toxicity is 1.5 to 3 times more toxic than in the dust phase (EFSA, 2012a). Additional research to underpin this assumption is recommended.

Note 11 The assessment of this xth percentile of the concentration of dust deposited on honey bees in this step 12 can be based on a tiered approach, starting with a very conservative generic approach (as suggested in Appendix J) and continuing if necessary with more realistic approaches based on the equipment to be used.

7.4 Risk assessment scheme for bumble bees and solitary bees

The proposed risk assessment scheme for bumble bees and solitary bees is only in a preliminary phase. There no reason to use a different type of scheme than that of honey bees. But, before it will be possible to run this scheme, additional research has to be done (see Chapter 5).

1 Collect details of the product and its pattern of use.

E.g. type of application (sprayed products, granular products, seed treatment, etc.), time of treatment in relation to crop flowering and any special directions for use (including mitigation), attractiveness of crop to non bumble bees and solitary bees, whether the active ingredient or metabolite is systemic and how the application fits within crop rotation, etc.

go to 2

2 Is exposure to bumble bees and solitary bees negligible (see Note 1)?

if yes classify risk as negligible
if no go to 3

3 Is the compound an insecticide, or an insect growth regulator or does the compound have insecticidal activity (see note 2)?

if yes go to 4
if no go to 12

Remark: A risk assessment for bumble bees and solitary bees is only carried out for insecticides, insect growth regulators or compounds with insecticidal activity, in contrast to honey bees where the risk is assessed for each compound.

4 How are the bumble bees and solitary bees exposed?

If active ingredient is applied as a spray go to 5
If active ingredient is applied as a granule or as seed treatment go to 14
If active ingredient does not fit in any of the above questions go to 19

Remark: it is possible that certain compounds tick more than one box. In that case all applicable routes have to be assessed.

Exposure to sprayed products

5 Assess whether the active ingredient can be systemically translocated in the plant to the pollen or the nectar or to the guttation droplets

If yes go to 6 and 8
If no go to 6

6 Establish adult oral and contact LD50 for bumble bees and solitary bees (see note 3).

Calculate the hazard quotient (HQ, see note 4) between the application rate and the LD50 toxicity values (g ha^{-1} /LD50 in µg per **gram** insect (bumble bees and solitary bees)

Assess possible longer term impacts on adult bumble bees and solitary bees using the endpoints of the LC50 study with *Apis* worker bees **as a surrogate** for bumble bees and solitary bees

Calculate the exposure toxicity ratio (ETR_{adults}) of the amount of residues (see note 5) that may be ingested by bumble bees and solitary bees in 1 day and the LC50 value.

Assess possible impacts on solitary bee and bumble bee larvae using *Apis* larvae test endpoint **as a surrogate** for solitary bee and bumble bee larvae.

Calculate the exposure toxicity ratio (ETR_{larvae}) of the amount of residues (see note 5) that may be ingested by bumble bee and solitary bee larvae in 1 day and the no observed effect level (NOEL).

Assess whether there is evidence of cumulative toxicity according to Haber's Law in the toxicity tests (see note 6).

if all HQs < 5 and ETR_{adult} < W and if ETR_{larvae} < Z (see note 7) and no evidence for cumulative toxicity go to 12
if one of the HQs ≥ 5 or ETR_{adult} ≥ W or ETR_{larvae} ≥ Z and/or evidence for cumulative toxicity go to 7

7 In case only the ETR_{adult} doses trigger further risk assessment or the compound has cumulative potential consider establishing adult LC50 values[11] for relevant species (see note 4) or **go directly to 10.**

Calculate the ratio (ETR_{adult}) between exposure (assessed by estimating the amount of residues that may be ingested by bumble bees and solitary bees in 1 day) and the LC50 value for bumble bees and/or solitary bees and assess the potential for cumulative toxicity.

if ETR_{adult} < X **and no evidence of cumulative toxicity go to 12**
if ETR_{adult} ≥ X or cumulative potential **go to 10**

8 Establish adult oral and contact LD50s for relevant for bumble bee and solitary bee species (see note 3).

Calculate the HQs_t between the exposure (see Note 8) by dust (x^{th} percentile concentration) of bumble bees and solitary bees (µg) and the LD50 in µg per **gram** bumble bee or solitary bee).

Assess possible longer term impacts on solitary bee and bumble bee larvae using the endpoints of the LC50 study with *Apis* worker bees **as a surrogate** for bumble bees or solitary bees.

Calculate the exposure toxicity ratio (ETR_{adults}) of the exposure by dust (x^{th} percentile concentration) of bumble bees and solitary bees and the LC50 value.

Assess possible impacts on solitary bee and bumble bee larvae using *Apis* larvae test endpoint **as a surrogate** for solitary bee and bumble bee larvae.

Calculate the exposure toxicity ratio (ETR_{larvae}) of the exposure by dust (x^{th} percentile concentration) of bumble bees and solitary bees and the no observed effect level (NOEL).

if all HQs < 5 and ETR_{adult} < W and if ETR_{larvae} < Z and no evidence for cumulative toxicity go to 12
if one of the HQs ≥ 5 or ETR_{adult} ≥ W or ETR_{larvae} ≥ Z and/or evidence for cumulative toxicity go to 9

[11] At this moment there is no dietary larval test other than for honey bees

9 In case only the ETR_{adult} doses trigger further risk assessment or the compound has cumulative potential consider establishing adult LC50 values[12] for relevant solitary bees and/or bumble bees or **go directly to 10.**

Calculate the ratio (ETR_{adult}) between the exposure by dust (x^{th} percentile concentration) of bumble bees and solitary bees and the LC50 value for relevant solitary bees and/or bumble bees.

if $ETR_{adult} < X$ **and no evidence of cumulative toxicity go to 12**

if $ETR_{adult} \geq X$ or cumulative potential **go to 10**

10 Assess the effects of the product in semi-field tests (see note 3).

Do results indicate minimal risk (no significant difference to control)?

if yes go to 12

if no go to 11

11 Assess the effects of the product in field tests[13] (see note 3).

Do results indicate minimal risk (no significant difference to control)?

if yes go to 12

if no go to 13

Analysis of uncertainty and risk categorisation

After completing the risk assessment based on data reflecting normal use of the product, the assessor should consider whether errors in measurements, or variation in conditions of use, might alter the conclusions.

12 Classify as **low risk** for bumble bees and solitary bees after analyzing the uncertainties.

13 Analyze uncertainties to confirm the conclusions. Consider whether specific risk management measures are possible

if yes reassess the potential risk

if no classify risk as high

Exposure via granular treatment and seed treatment

14 Assess whether the active ingredient can be systemically translocated in the plant to the pollen or the nectar or to the guttation droplets.

Assess whether bumble bees and solitary bees can be exposed to dust during application

If the answer is yes to both questions go to 15 and 17

If exposure is only to dust go to 15

If compound is only systemic go to 17

If the answer is no to both questions go to 12

15 Establish adult oral and contact LD50s for bumble bees and solitary bees (see note 3).

Calculate the HQs_t between the exposure (see Note 8) by dust (x^{th} percentile concentration (see note 9) of bumble bees and solitary bees (µg) and the LD50 in µg per **gram** bumble bee or solitary bee).

Assess possible longer term impacts on solitary bee and bumble bee larvae using the endpoints of the LC50 study with *Apis* worker bees **as a surrogate** for bumble bees or solitary bees.

Calculate the exposure toxicity ratio (ETR_{adults}) of the exposure by dust (x^{th} percentile concentration) of bumble bees and solitary bees and the LC50 value.

Assess possible impacts on solitary bee and bumble bee larvae using *Apis* larvae test endpoint **as a surrogate** for solitary bee and bumble bee larvae.

Calculate the exposure toxicity ratio (ETR_{larvae}) of the exposure by dust (x^{th} percentile concentration?) of bumble bees and solitary bees and the no observed effect level (NOEL).

if all HQs < 5 and $ETR_{adult} < W$ **and if** $ETR_{larvae} < Z$ **and no evidence for cumulative toxicity go to 12**

[12] At this moment there is no dietary larval test other than for honey bees

[13] Tests should be carried out according to good agricultural practice.

if one of the HQs ≥ 5 or ETR_{adult} ≥ W or ETR_{larvae} ≥ Z and/or evidence for cumulative toxicity go to 16

16 In case only the ETR_{adult} doses trigger further risk assessment or the compound has cumulative potential consider establishing adult LC50 values[14] for relevant solitary bees and/or bumble bees or **go directly to 10.**

Calculate the ratio (ETR_{adult}) between the exposure by dust (x^{th} percentile concentration) of bumble bees and solitary bees and the LC50 value for relevant solitary bees and/or bumble bees.

if ETR_{adult} < X **and no evidence of cumulative toxicity go to 12**

if ETR_{adult} ≥ X or cumulative potential **go to 10**

17 Establish adult oral and contact LD50s for relevant bumble bee species and solitary bee species (see note 3).

Calculate the ETR_{acute} between the amount of residues (see note 5) that may be ingested by bumble bees and solitary bees in 1 day via guttation fluid, contaminated pollen and/or contaminated nectar (Daily intake per bumble bee and solitary bee (µg)/LD50 in µg per **gram** bumble bee or solitary bee).

Assess possible longer term impacts on solitary bee and bumble bee larvae using the endpoints of the LC50 study with *Apis* worker bees **as a surrogate** for bumble bees and solitary bees.

Calculate the exposure toxicity ratio (ETR_{adults}) of the amount of residue (see note 5) that may be ingested by a bumble bee and a solitary bee in 1 day via guttation fluid, contaminated pollen and/or contaminated nectar and the LC50 value.

Assess possible impacts on bumble bee and solitary bee larvae using *Apis* larvae test endpoint **as a surrogate** for bumble bee and solitary bee larvae.

Calculate the exposure toxicity ratio (ETR_{larvae}) of the amount of residues (see note 5) that may be ingested by a bumble bee and solitary bee larvae in 1 day via guttation fluid, contaminated pollen and/or contaminated nectar in 1 day and the no observed effect level (NOEL).

if all ETR_{acute} **< Y and** ETR_{adult} **< X and if** ETR_{larvae} **< Y and no evidence for cumulative toxicity go to 12**

if one of the ETR_{acute} **< Y or** ETR_{adult} **≥ X or** ETR_{larvae} **≥ Y and/or evidence for cumulative toxicity go to 18**

18 In case only the ETR_{adult} doses trigger further risk assessment or the compound has cumulative potential consider establishing adult LC50 values[15] for relevant species (see note 3) or **go directly to 10.**

Calculate the ratio (ETR_{adult}) between the amount of residues that may be ingested by bumble bees and solitary bees in 1 day via guttation fluid, contaminated pollen and/or contaminated nectar and the LC50 value for the species.

if ETR_{adult} < X **and no evidence of cumulative toxicity go to 12**

if ETR_{adult} ≥ X or cumulative potential **go to 10**

Other compounds not covered in the steps before

19 For novel application methods, specific risk assessment methods may need to be developed (e.g termite baits). As a minimum, reports of acute oral and contact tests and a chronic toxicity test shall be submitted.

Notes

Note 1 According to the latest version of the draft on regulatory requirements for active substances (SANCO, 2011) for seed treatments there needs to be a consideration of the risk from drift or dust during time of drilling the treated seed and for granules and slug pellets there needs to be a consideration of the risk from drift of dust during application (exposure of bees visiting neighbouring plants). If an active substance is systemic and to be used on seeds, bulbs, roots, applied directly to soil (e.g. sprayed on to soil, granules/pellets applied to soil, irrigation water) or directly into the plant (e.g. stem injection),

[14] At this moment there is no dietary larval test other than for honey bees

[15] At this moment there is no dietary larval test other than for honey bees

then the risk to bees foraging those plants should be assessed, including the risk deriving from pesticide residues in nectar, pollen and water, including guttation.
Examples of when exposure of bees is not possible: food storage in enclosed spaces, non-systemic dipping treatments for transplanted crops and bulbs, wound sealing and healing treatments, and use in glasshouses without bumble bees and solitary bees as pollinators.

Note 2 Data for the non-target arthropods could be used for assessing the potential insecticidal activity of a compound. For most of the compounds the two standard non target arthropods are tested (*Typhodromus pyri* and *Aphidius rhopalosiphi*). When the quotient of the application rate multiplied by a MAF factor and the LR50 is greater than 2, the compound could be considered as having insecticidal activity. In addition efficacy studies with other insects or studies carried out with insects in the screening process could be another source for assessing potential insecticidal activity.

Note 3 In Chapter 4 an overview of available tests is presented for bumble bees and solitary bees. The development of standardized contact and oral toxicity test, semi field and field tests for bumble bees and solitary bees has yet to be completed and ring tested. There is a need to improve the testing protocols concerning bumble bees and solitary bees, in particular to better address the chronic risk and the identification and measurement of sub-lethal effects (e.g. effects on memory, learning capacity, orientation) to be used in the risk assessment.
There is no equivalent EPPO 170 or OECD 75 guideline for use in testing bumble bees and solitary bees in semi-field or field studies; however, information from some field studies conducted on non target arthropods can be used for the risk assessment for bumble bees and solitary bees. It is recommended when designing new semi fields studies to use EPPO 170 as a general guide. Relevant endpoints are mortality, foraging and reproductive success.
As for tests with honey bees, the tests for bumble bees and solitary bees shall provide the EC_{10}, EC_{20}, EC_{50} (or an explanation if they cannot be estimated) together with the NOEC. Sub-lethal effects, if observed, shall be reported.
Probably a single semi field test or single field test will not give the answer to whether the compound is safe to use in agriculture. A field study should have sufficient power to detect the effects above the suggested thresholds for the magnitude of effects.
A definitive proposal of the number of studies to be carried out and how severe the test circumstances have to be, will be established as part of the development of the guidance document for honey bees.

Note 4 For bumble bees and solitary bees it is proposed to use the same trigger for the hazard quotient of 50 as is used in the honey bee risk assessment scheme. But for bumble bees and solitary bees the unit is per gram bee instead of per bee. Therefore, the HQ trigger is 5 instead of 50 (body weight of a bee is approximately 100 milligrams). Additional research to underpin this assumption is recommended.

Note 5 Residue levels in honey and pollen. According to the latest version of the draft on regulatory requirements for active substances (SANCO, 2011) residue levels in honey and pollen should be reported. Residue studies are normally required: when a plant protection product is used during or shortly before blossom of the crop or when a plant protection product is used before blossom and the active substance used has a low degradation rate and/or is systemic and when these flowering crops are used to produce pure blossom honey.
For environmental risk assessment, tests should preferably be conducted in semi-field or under worst case field conditions, using the plant protection product in accordance with the critical GAP. Immediately after the end of blossoming honey and pollen should be analyzed. Results from at least three trials performed at different geographical locations should be provided.
To be consistent with the exposure assessment aim, the exposure estimate should be equal to or higher than a x^{th} percentile case.

Note 6 Guidance for calculating whether an active ingredient shows evidence of cumulative toxicity according to Haber's Law can be found in Chapter 4.

Note 7 The risk from short-term exposure (approximately 10 days) to contaminated food ad libitum is based on an exposure toxicity scenario. Preliminary thoughts for trigger values are 1 when the toxicity endpoint is the NOEC and 10 when the endpoint is a LC50 value. In case of using the honey bee toxicity data as a surrogate for predicting the risk for bumble bees and solitary bees, the preliminary thought is to use a ten times more conservative approach than for honey bees. A definitive proposal for

the values X, Y, Z and W in the bumble bee and solitary bee scheme will be established as part of the development of the guidance document (see also appendix R).

Note 8 It is believed that in case of equal amounts of exposure that exposure of the compound solved in either water or a solvent could be considered as more worst case than exposure of the compound in a dry phase (e.g. dust). There is some circumstantial evidence that this assumption could be true. In studies with normal humidity and high humidity, honey bee mortality after dust exposure was greater under high humidity circumstances (Mazaro et al., 2011). Data submitted for the peer review of the pesticide risk assessment of the active substance thiamethoxam shows that the acute toxicity data for oral exposure as well as contact exposure are comparable for a liquid formulation and a dust formulation (in liquid phase the toxicity is 1.5 to 3 times more toxic than in the dust phase (EFSA, 2012a). Additional research to underpin this assumption is recommended.

Note 9 The assessment of this x^{th} percentile of the concentration of dust deposited on bumble bees and solitary bees in this step 15 can be based on a tiered approach, starting with a very conservative generic approach (as suggested in Appendix J) and continuing if necessary with more realistic approaches based on the equipment to be used.

8 CHAPTER 8: RECOMMENDATIONS AND CONCLUSIONS

For the development of robust and efficient environmental risk assessment procedures it is crucial to know what to protect, where to protect it and over what time period. The methodology of definition of specific protection goals follows the approach outlined in the Scientific Opinion of EFSA (2010). The Working Group identified pollination, hive products (for honey bees only) and biodiversity (specifically addressed under genetic resources and cultural services) as relevant ecosystem services. It is suggested to define the attributes to protect for the survival and development of colonies and effects on larvae and honey bee behaviour as listed in regulation (EC) No 1107/2009. In addition it is proposed to also include abundance/biomass and reproduction because of their importance for the development and long-term survival of colonies.

The magnitude of effects was defined as negligible if the natural background mortality, compared to controls, is not exceeded. An effect is defined as small if the natural background mortality is increased for example by a factor of 2. Further work is needed to give recommendations on the deviation from the controls up to which an effect is still considered negligible. The current methods of field testing would need major improvements in order to detect for example an increase in daily mortality of foragers by 10% with high statistical power. Based on expert judgement it was considered that a small effect could be tolerated for some days without putting the survival of a hive at risk. However, it is not clear up to what extent the strength of the colony would be affected. Further research (modelling) is proposed to clarify this question and to revise the proposal for the magnitude and temporal scale of effects.

The final decision on protection goals needs to be taken by risk managers. There is a trade-off between plant protection and the protection of bees. The effects on pollinators need to be weighed against increase in crop yields due to better protection of crops against pests. The overall level of protection also includes the exposure assessment goals. Decisions need to be taken on how conservative the exposure estimate should be and what percentage of exposure situations should be covered in the risk assessment. The first aspect of the spatial statistical population is the total area to be considered (e.g. the whole EU, one of the regulatory zones North-Centre-South or a Member State). In view of the terms of reference, we propose to consider each of the regulatory zones North-Centre-South as the total area for all specific protection goals (SPGs). A second aspect of the spatial statistical population is the location of the spatial units (individual bees, colonies or populations) in the landscape in relation to the application of the substance. It is proposed that the risk assessment focuses at field scale to avoid ‘dilution’ of the spatial population with a large fraction of unexposed hives, for example.

The current risk assessment for honey bees relies on a Hazard Quotient (HQ) approach (application rate/LD50) and semi-field and field tests. It is particularly difficult to ascertain whether a specific exposure percentile is achieved in semi-field and field studies. It is recommended to design a flow chart for checking whether exposure in the semi-field or field studies was indeed higher than that corresponding to a certain percentile. Factors that may be included are the crop and its developmental stage, the dosage, measures ensuring that bees are coming into contact with the compound/formulation, weather conditions, and for instance the generation of guttation droplets by the crop.

Residues in different environmental matrices and bee products were combined with estimates of exposure of different categories of bees. Highest concentrations of residues were found after spray treatments in pollen and nectar. Residues in guttation droplets showed a wide variability due to the number of parameters known to influence guttation production (environmental conditions, crop type, growth stage, etc.). A potentially high exposure was highlighted for bees in some crops (e.g. maize). Exposure to dust drift from sowing treated seeds was identified as a relevant exposure route.

The exposure of different categories of bees from different sources and for different application techniques suggest that the potential risk from oral uptake was highest for forager bees, winter bees and larvae through pollen, nectar and water. The exposure of nurse bees is by a combination of pollen and nectar. Larvae in contact with wax and flying foragers, drones, queens and swarms intercepting droplets and vapour in/out field were found to be the most exposed categories of bees via contact and inhalation exposures, respectively.

Worker bees, queens and larvae of bumble bees and adult females and larvae of solitary bees were considered the most exposed bee categories via oral uptake. Larvae of solitary and bumble bees consume large mass provisions with unprocessed pollen thus, compared with honey bee larvae, they are more exposed to residues in pollen. Moreover, bumble bee and solitary bees may be exposed to a larger extent via contact to nesting material (soil or plants) compared to honey bees suggesting the need for a separate risk assessment for bumble bees and solitary bees.

For the ranking of bees, the inclusion of multiple exposures with appropriate weights would need to be done with a modelling or scenario-based approach that was not available in the current assessment. It was therefore recommended that the categories of bees which represent the worst-case exposure scenarios through multiple exposures are further assessed (e.g. honey bee nurses) and that those categories which highlighted potential but unknown exposures through consumption of water and inhalation of vapour in/out field are further analysed with more studies. Further research is recommended on the testing of the presence and fate of residues (e.g. in bee relevant matrices and in-hive following spray and dust applications) and on the development of reliable exposure models.

The overview of the available studies on sub-lethal doses and long-term effects of pesticides on bees highlighted gaps in knowledge and research needs in the following areas: more toxicological studies to be performed in bees for a wider range of pesticides on both adults and larvae including sub-lethal endpoints, including also contact and inhalation routes of exposure. Few studies were conducted with non-*Apis* bees, considering endpoints such as fecundity (e.g. drones production in *Bombus* and cell production rate in solitary bees), larvae mortality rate, adult longevity and foraging behaviour. The use of micro-colonies in bumble bees appears to be well-suited to measure lethal and sub-lethal effects of pesticides with low doses and long-term effects.
Because of the specific toxicokinetic profile of bees compared with other insects, it is recognised that toxicokinetic data can provide useful information on the potential biological persistence of a pesticide which, in some cases, could have effects after continuous exposure that maybe more marked compared with their short-term effects. The integration of toxicokinetic knowledge and low (sub-lethal) dose effects generated from laboratory and field studies in the hazard identification and hazard characterisation of pesticides in *Apis* and *non-Apis* bees can provide a better understanding of short-term and long-term effects. It is therefore concluded that the conventional regulatory tests based on acute toxicity (48 to 96 h) are likely to be unsuited to assess the risks of long-term exposures to pesticides.

A testing protocol and mathematic model, based on Haber's Law have been developed as a simple prioritisation tool to investigate the potential effects after repeated exposure to single pesticides using mortality data. However, a number of assumptions inherent to the model raise uncertainties. The protocol and model needs further validation in the laboratory and to be tested for sub-lethal endpoints in adult and bee larvae. Finally, combining basic toxicokinetic data for an active substance and its metabolites, such as the half life, will also provide more precise estimates on the potential of bioaccumulation. In the case of potential persistence of the active ingredient, half life of the parent compound and its metabolites should be determined in larvae, newly emerged bees and foragers.

The EU risk assessment of pesticides on bees includes the determination of oral and contact acute toxicity (LD50) of adult honey bees (*Apis mellifera*) following EPPO guidelines 170 and OECD 213 and 214. Several exposure routes of pesticides are not evaluated in laboratory conditions: the intermittent and prolonged continuous exposuresof adult bees, exposure through inhalation and the

exposure of larvae. Likewise, the effects of sub-lethal doses of pesticides are not evaluated in conventional testing for now.

The observation period in acute studies should be extended beyond 96 hours if a further increase of effects is expected. It is proposed to include additional prolonged adult toxicity tests at the first tier level, both for adult bees and larvae (7-10 day exposure).

Sub-lethal effects should be taken into account and observed in laboratory studies. The importance of investigating sub-lethal effects was also demonstrated in a recent article of Henry et al. 2012[16]. It should be investigated further how to integrate specific laboratory tests in the first tier testing in future. Examples of potential laboratory methods to investigate sub-lethal effects include testing of *Bombus* microcolonies to investigate effects on reproduction, proboscis extension reflex test for neurotoxic effects and homing behaviour for effects on orientation and navigation. Further research is needed in order to integrate the results of these studies in the risk assessment scheme.

The development of molecular markers should be evaluated to determine if they can be used to predict effects such as critical immune system changes and the potential for interactions between active ingredients under real use conditions.

The available protocols for the testing of solitary bees are suitable to study the oral and contact toxicity in adults and larvae for several species of solitary bees (*Megachile rotundata*, *Osmia* spp.) but they need to be ring tested. More studies are necessary to compare the susceptibility of honey bees with that of other non-*Apis* species in order to see to which extent honey bee endpoints cover also non-*Apis* bees.

For semi-field testing (cage, tunnel or tent tests) currently three test guidelines: are available: the EPPO 170 (4), the OECD 75 brood guidance document, and the Oomen et al. (1992) test. Semi-field testing appears to be a useful option for higher tier testing. Nevertheless, weaknesses have been identified for each of the test guidelines e.g. the limited size of crop area, the impossibility to evaluate all the possible exposure routes of the systemic compounds used as seed- and soil-treatments (SSST), the limited potential to extrapolate the findings on larger colony sizes used in field studies or the relatively short timescale (one brood cycle), the long-term assessment of brood. It is recommended to add the current state of scientific knowledge on a number of issues to the existing guidelines (e.g. more detailed description and categorization of behavioural assessments, investigation of sub-lethal and delayed effects, inclusion of assessment of disease status of the colonies before and after exposure etc.) and to develop methodologies adapted to pesticides applied as SSST, including all potentially relevant exposure routes (e.g. for dust drift and guttation). Consideration should be given to extending studies where significant exposure is likely to occur over a long period. Results should be analysed with appropriate statistical methods.

Field testing currently follows the EPPO 170 (4). A scientific assessment of these guidelines showed that it has several major weaknesses (e.g. the small size of the colonies, the very small distance between the hives and the treated field, the very low surface of the test field), leading to uncertainties concerning the real exposure of the honey bees. The guideline is better suited to the assessment of spray products than to that of SSST.

No formalized semi-field or field test guidelines are available for bumble bees and solitary bees and further work is required to develop guidelines, including the minimum field size, number of colonies or nesting females per treatment, methodology for dead bee assessments and foraging assessments and agreement of appropriate approaches for determining colony development (for bumble bees). Effects

[16] EFSA received a request from the European Commission for a scientific statement on the articles of Henry et al. 2012 and Whitehorn et al. 2012. It is expected that the statement of EFSA will be issued at the end of May 2012.

on reproduction of bumble bees following exposure to imidacloprid were observed in a recently published study (Whitehorn et al. 2012)[17].

There is a general need to improve the statistical power of the tests considered in this opinion, mainly those run in semi-field and field conditions.

Future research is recommended to improve laboratory, semi-field and field tests (e.g. extrapolation of the endpoints in first tier to the colony/forager effects, extrapolation of the toxicity between dust and spray, extrapolation of laboratory based *Bombus* micro colonies to *Apis* and solitary bees).

Separate risk assessment schemes are proposed, one for honey bees and one for bumble bees and solitary bees. In the first tier it is proposed to include toxicity testing that covers a longer period of exposure (7 to 10 days) for adult bees as well as bee larvae. Both life stages can be exposed for more than one day and this risk was not covered by the standard OECD tests (213 and 214) for oral and contact exposure. Currently there is insufficient evidence that toxicity following extended exposures can be reliably predicted from acute oral LD50 data. It is also proposed to investigate for each compound whether there are any indications of cumulative effects. A new method to detect cumulative toxicity is proposed based on the Haber`s Law. If there is an indication that a compound is a cumulative toxin then this needs further evaluation since the potential effects of continuous or repeated exposure to low doses may be underestimated.

If the LD50 for the adult bee is greater than 100 µg/bee and there is no evidence of cumulative toxicity in larvae in the 7 day larval test (Aupinel et al., 2007, 2009) then no further larval tests are required and the NOEL (chronic 7 day larva) is used in risk assessment. In all other cases (including IGR) an Oomen et al. (1992) study type is required in first tier to evaluate effects during metamorphosis and to integrate brood care behaviour of the adult bees and the lower NOEL from the Aupinel and Oomen is used in the scheme. If higher tier studies need to be done because adult toxicity tests triggered them, it would be recommendable to include observations on the brood and lifespan of adults.

The scheme for bumble bees and solitary bees is based on less well defined steps and toxicity tests. The scheme uses data for honey bees as surrogate input. The development of standardized contact and oral toxicity test, semi field and field tests for bumble bees and solitary bees has yet to be completed and ring tested. There is a need to improve the testing protocols concerning bumble bees and solitary bees, in particular to better address the chronic risk and the identification and measurement of sub-lethal effects (e.g. effects on memory, learning capacity, orientation) to be used in the risk assessment. The primary concerns for bumble bee and solitary bee species were considered to be from insecticides, pesticides with insecticidal activity and insect growth regulators and therefore the risk assessment proposed is primarily for these modes of action. However, the honey bee scheme has to be applied for each compound unless the risk can be characterized as negligible. Note that when exposure to bees is classified as negligible the exposure to bumbles bees and solitary bees could still be possible.

Pesticides containing a number of active ingredients are frequently applied sequentially or as mixtures such as tank mixes, and there is a consensus in the field of mixture toxicology that the customary chemical-by-chemical approach to risk assessment is too simplistic. At low levels of exposure concentration addition has been observed more often than synergistic or antagonistic effects for mixtures of pesticides with a common mode of action and independent action (response addition) has been observed for compounds with a different mode of action. In some cases synergistic and antagonistic effects have also been observed.

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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. The mechanisms of such interactions involved inhibition or induction of either detoxification enzymes (cytochrome P-450) or transporters which then enhanced the toxicity of the mixture decreasing the LD50. 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 the case of synergism which can be predicted based on the mode of action of the chemical classes involved (e.g. EBI fungicides and insecticides), and in the absence of existing data on toxicity of the mixture, it is recommended to design full dose-response studies in adult bees and larvae for mixtures of potential synergists. These should take into take into account the dose dependency of the synergy, the magnitude of the interaction at concentrations of environmental relevance as well as both the maximum potentiating factor of the synergist and the concentrations for which no potentiating factor occur in the dose response curve. Such statistically-sound dose response data will provide a basis to derive benchmark doses and their limit as suggested by EFSA's scientific committee. This flexible approach would allow quantitative protection goals to be achieved (e.g. specific effect size for lethality or for a sub-lethal effect depending on the protection goal and the aim of the risk assessment). Further work is also required to identify the molecular basis of interactions between environmentally realistic exposure to pesticides and the range of honey bee diseases (fungi, bacteria and viruses) to determine whether and how these may be included in risk assessment.

Further research needs were identified in the following areas in order to improve future risk assessments for bees:

1. Research aimed at a better understanding of the mechanisms of action and detoxification/metabolization of the pesticides to which bees are exposed in the field.

- Toxicokinetics and toxicodynamics of chemicals (both parent compounds and metabolites) in bees (absorption, distribution, metabolism and excretion), and receptor-toxic interactions. Studies should aim at creating models able to incorporate different exposure paths for which information such as half-life and residue levels in exposed bees would be needed.

- Testing the interactions between genes and enzymes involved in the detoxification processes.

- Studying the impact of the dysfunction of the detoxification enzymes on the metabolism of endogenous compounds such as pheromones, and implication at the colony level (effects on communication, learning performance, etc.).

2. Research aimed at developing and standardizing precise analytical methods for measuring the exposure of bees to pesticides (e.g., using relevant LOD and LOQ), for all the matrices (nectar, pollen, bees, wax, water, dust, etc.)

3. Research aimed at developing tests for studying the toxicity of pesticides for bees:

   - at individual level: chronic and sub-lethal toxicity for adult workers, effects on the fecundity and longevity of the queens and fecundity of the drones, effects on the development of pre-imaginal stages (eggs, larvae and nymphs) including brood feeding and heating.

   - at colony level: chronic and sub-lethal toxicity, long-term effects studies

Precise and replicable protocols[18], and methods appropriate for statistical evaluation, should be developed for laboratory, semi-field and field testing. Criteria for checking the quality of laboratory, semi-field and field testing should be established.

Concerning honey bees (*Apis mellifera*) including about ten subspecies (= geographic races) in Europe, inter-subspecies variability of the pesticides' effects should be studied, at least for those races that are the most used by the European beekeepers.

Appropriate exposure scenarios and modeling approaches should be developed.

a) Laboratory

   i. Prolonged exposure

      - Development of standardized protocols for measuring the effects of prolonged exposure.

      - Evaluation of the data generated by prolonged exposure studies in adults based on intermittent *versus* continuous exposures, in order to determine the most appropriate test design.

   ii. Sub-lethal effects

      - Development of standardized protocols for measuring sub-lethal toxicity on the behaviour, physiology and neurophysiology of bees. The protocols should make specific reference to variability factors such as the age of the tested bees, the laboratory conditions (i.e. temperature and hygrometry), the bee subspecies, their nutritional and health status, etc.

      - Research on the relationship between effects on individuals and effects on the colony should be done.

      - The relative sensitivity of test methods should be measured and linked to endpoints at the first tier on the colony/forager level effects (e.g.: PER, homing behaviour and *Bombus* microcolony studies).

      - Suitability of laboratory based *Bombus* micro colonies for evaluating reproductive effects of products should be checked, both in their extrapolation to *Apis* and solitary bees and how to integrate the results into a risk assessment scheme.

      - Development of molecular markers of toxic effects to enable prediction of potential sub-lethal effects such as immune system changes, potential interactions between products and effects in colony under real use conditions.

[18] For a review look at: Haynes 1988; Thompson 2003; Desneux et al. 2007; Decourtye and Devillers 2010.

- Further study of the histological modifications caused by pesticides on bees to enable prediction of potential sub-lethal effects under real use conditions

- Determination of thresholds that induce shifts in bee activity (e.g. sub-lethal effects induce a shift of nurses into foragers). Model and make scenarios on all the possible cascade effects on the colony.

- Analysis of interactions and synergisms (e.g. agrochemicals and veterinary products with diseases) at low doses.

iii. Larvae toxicity tests

- Study the behaviour and physico-chemical characteristics of different toxic standards used in larvae toxicity tests in order to optimise exposure.

- Develop a standardized protocol for measuring toxicity in bumble bees and solitary bees.

b) Semi-field

i. Update the current protocols in accordance with state-of-the-art scientific literature on honey bees, for including precise descriptions of the behavioral and other sub-lethal and delayed end-points, for the individuals and for the colony.

ii. Specific guidance on the assessment of different behavioral endpoints should be provided, in particular concerning brood development and colony reproduction.

iii. Develop methodologies adapted to pesticides applied as SSST.

iv. Develop a standardized protocol for bumble bees and solitary bees in semi-field conditions.

c) Field

i. Development of standardized protocols for:

- a generic test, based on the principles of the EPPO 170 guideline but with further improvements including specific guidelines for bumble bees and solitary bees.

- some specific tests for assessing the effect of a pesticide on specific behavioral endpoints, such as, for example, the homing flight and orientation, the foraging behavior, etc.

ii. Precise protocols should be developed for field testing of SSST products. These protocols should include precise descriptions of the behavioral and other sub-lethal and delayed end-points, for the individuals and for the colony.

iii. Automatic methods for measuring the activity of honey bee colonies should be developed or improved, e.g. for measuring the number of honey bees leaving the colony for foraging, the number of foraging honey bees returning to the colony and (by difference) the number of foraging honey bees eventually lost outside the hive, precise methods of measuring mortality of foragers and in-hive bees.

iv. Comparison should be done among the sensitivity to a given pesticide of small (10,000 individuals) colonies versus normal colonies (30,000 – 40,000 individuals).

v. Protocols for post-marketing studies to monitor the effects of registered pesticides should be developed.

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APPENDICES

A. OPTIMAL NUMBER OF POLLINATORS PER CROP

Apis mellifera

Crop	Cultivar	No of hives/ha*	References
Apricot	A. Errani, Aurora, Goldric	7–8	Contessi (2005)
	All others	5–6	Contessi (2005)
Cherry	Mean size plants	4–6	Contessi (2005)
	Large size plants	8–10	Contessi (2005)
Kiwi	All	8–10	Contessi (2005)
Apple	Red Delicious	7–8	Contessi (2005)
	All others	6–7	Contessi (2005)
Pear	Abate Fetel, Decana	8–10	Contessi (2005)
	All others	6–7	Contessi (2005)
Peach	All	4–8	Contessi (2005)
European plum	All	7–8	Contessi (2005)
Chinese–Japanese plum	All	8–10	Contessi (2005)
Strawberry (field)	All	2–4	Contessi (2005)
Strawberry (tunnel)	All	2–4 nukes	Contessi (2005)
Raspberry	All	2–4	Contessi (2005)
Blueberry	All	2–4	Contessi (2005)
Alfalfa		6–8	Contessi (2005)
Clover (*Trifolium*)		4–6	Contessi (2005)
Others Leguminosae		2–4	Contessi (2005)
Cucurbitaceae		4–6	Contessi (2005)
Brassicaceae		3–4	Contessi (2005)
Compositae		3–4	Contessi (2005)
Sunflower		8–10	Contessi (2005)

Osmia spp.

Bee species	Crop	Nesting females/ha*	References
O. lignaria	Almond	740	Torchio (1991)
	Apple	650	Torchio (1985)
O. cornifrons	Apple	550	Maeta (1990)
O. cornuta	Almond	750	Bosch (1994)
	Apple	530	Vicens and Bosch (2000)

*The numbers given are for single species and not for a mixed population of pollinators.

Megachile rotundata

In the USA 100 000–150 000 bees/ha (or 40 000–70 000 females considering the sex ratio) are used for alfalfa seed production (Pitts-Singer and Cane, 2011).

Many bee species have been introduced for crop pollination around the world due to their importance as pollinators. However, the number of documented accidental introductions is far greater than the number of intended introductions (Vergara, 2008). Introduced honey bees could potentially compete for floral resources with native bees and cause reductions in survival, growth or reproduction in these bees. Based on the few studies that have quantified the impact of honey bees on native bee competition, it is not possible to draw any definite conclusion regarding the real impact of honey bees on wild bees, and no experiment has clearly demonstrated long-term reductions in populations of native organisms (Goulson, 2003; Vergara, 2008; Shavit et al., 2009). Moreover, resource division in the pollinator communities is quite common due to the different biological traits among the bee taxa,

including floral preferences and specialisation, flight season, daily activity, foraging range, life cycle. In recent years, the decline of wild pollinators, combined with the poor crop yields in self-incompatible cultivars, has raised the necessity to introduce domestic pollinators in crops (e.g. Biesmeijer et al., 2006). The honey bee, *Apis mellifera*, is used throughout the world on a vast array of cultivated plants and several bumble bee species, *Bombus* spp., are used in greenhouses for pollination of tomato and other crops. Among solitary bees, a few species were developed as crop pollinators, including the alfalfa leaf-cutting bee, *Megachile rotundata* (mostly used for alfalfa pollination), several species of mason bees, *Osmia* spp. (mostly managed for fruit tree pollination), and the alkali bee, *Nomia melanderi*, used for alfalfa pollination. The number of pollinators necessary to provide optimum pollination for a hectare of crop depends on many factors, including the crop species or cultivar, and the ratio and distribution of polliniser trees in the field. It also varies from region to region and from year to year, depending on the weather, the presence of wild pollinators and apiaries in the zone. While the introduction of managed bees in-field assures good pollination service (quality and quantity in crop production) (McGregor, 1976; Free, 1993), evidence also supports the positive role of pollinator diversity out-field in maintaining plant community diversity (Fontaine et al., 2006).

B. CRITICAL THRESHOLD OF BEE MORTALITY IN THE MONITORING STUDIES IN ITALY (PORRINI ET AL., 2002, 2003)

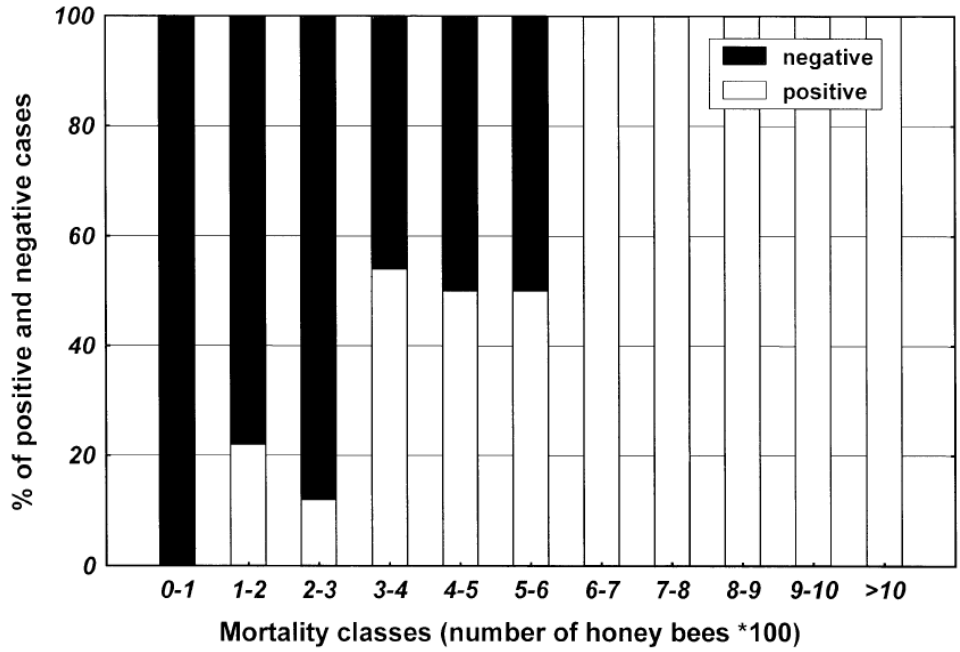
For about 20 years the research group of the Institute of Entomology of the University of Bologna has been studying the relationship between honey bees and pesticides through monitoring systems. The Italian monitoring system was applied not only for proving potential bee poisoning risks by the use of pesticides, but also for determining the degree of environmental contamination due to plant protection products. Since 1980, this strategy has been applied in more than 35 towns in Italy and more than 400 monitoring stations have been installed to cover a total territory of 2 800 km^2. Each monitoring station consists of two beehives equipped with collection cages for dead bees ("underbasket"). Once a week, colonies are checked and the number of dead bees is recorded. When the mortality rate exceeds the critical threshold (250 bees/week/station), laboratory analyses are carried out.

Various experiments have been undertaken for the purpose of determining a critical threshold of mortality that may be assessed using underbaskets. A study takes into account observations made in non-contaminated areas where this type of trap was shown to capture weekly up to 2.5 % (175 dead bees per hive) of the assumed maximum natural mortality of about 1 000 bees/day.

Six monitoring stations, each comprising two hives, were posted in the township of Castenaso (province of Bologna). In the months of April and May 1997, the dead bees found in the baskets positioned in front of the hives were retrieved and analysed weekly. The aim of the study was to identify the level of mortality at which pesticide residues began to appear; this level would thus be considered as the threshold marking the boundary between natural and induced mortality.

During the experiments, 48 samples of dead bees were collected and analysed. Seventeen samples exceeded the critical threshold hypothesised of 350 dead bees per week per station (the sum of two hives). Fourteen (82.3 %) were found to be positive, i.e. they contained at least one residue of a compound, whereas only 19.3 % of the samples below the threshold of 350 dead bees were found to be positive. Of the latter samples, 66.6 % fell within the bracket of mortality ranging from 300 to 350 dead bees (Figure 1). On the basis of these findings, therefore, it can be deduced that the critical threshold of mortality in a station comprising two hives is 300–350 dead bees per week.

However, in some cases it may also be useful to analyse bee specimens that do not reach the critical threshold of mortality as they can provide evidence of active principles harmless for bees but dangerous for the environment. For this reason the critical threshold was lowered to 250 dead bees for two colonies per week.


100
80
60
40
20
0
% of positive and negative cases
negative
positive
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
>10
Mortality classes (number of honey bees *100)

C. OVERVIEW OF SOLITARY BEE BIOLOGY

The superfamily Apoidea comprises more than 20 000 species, ~700 genera and 10 families. The Apoidea are divided into two groups: the bees, or Apiformes, and the sphecoid wasps, or Spheciformes (Table C1). Bees are very diverse and abundant, with more than 16 000 species worldwide. They differ from nearly all wasps in their dependence on pollen collected from flowers as a protein source to feed their larvae and probably also for ovarian development by egg-laying females (Michener, 1974, 2000). The vast majority of the genera and species of the bees are solitary (more than 85 %), that is, each female builds and provisions her own nest without assistance from other bees and has no contact with the offspring after the eggs are laid (Batra, 1984). Numerous definitions have been proposed to distinguish between social and solitary bees, but recent information has shown that hard and fast distinctions cannot always be made. In fact, if the honey bee represents the ultimate in social organisation among bees, there are many species that represent an intermediate condition. For example, the species of the subfamily Halictinae display all stages of social behaviour from solitary to highly social (Stephen et al., 1969).

Table C1: Classification of the superfamily Apoidea

Series	Family
Apiformes	Stenotritidae
	Collectidae
	Andrenidae
	Halictidae
	Mellittidae
	Megachilidae
	Apidae
Spheciformes	Ampulicidae
	Crabronidae
	Sphecidae

Bees live everywhere, showing the greatest abundance and diversity of species in semi-arid, warm temperate climates. In temperate regions most species of bees have only one generation a year and spend the winter (and much of the rest of the year) as prepupae in their natal cells. Other bees overwinter as unemerged adults within natal cells (*Andrena* spp., *Osmia* spp., some *Antophora* and *Megachile*). In a number of species which have two or more complete generations, adult females of the last generation mate and overwinter in special hibernacula (*Bombus*, some halictines). Both sexes of the Xylocopinae overwinter as emerged adults and mate in the spring. Mating is usually the first activity of adult bees once they have emerged from the nest, and in most cases it takes place at or near the nesting site, often on a substrate (soil, flower, leaf, etc.). Unlike drones, males of most bees can copulate numerous times. After mating, most species of bees begin searching for suitable niches in which to nest. It is during this period that the most dispersal occurs; in fact, females may travel a few metres to a few kilometres in search of a suitable nesting site. Most bee nests consist of more than cells, being burrowed in the soil or in wood (Stephen et al., 1969; Michener, 2000). The majority of bees that nest in wood utilise existing insect burrows or hollow stems. The cells can be lined or unlined and each cell can be separated by partitions with leaf pieces (*Megachile*), resin (*Heriades*) or mud (*Osmia*). A cell serves to protect the delicate, immature stages and in most cases the food, or the growing larva. Provisions stored by bees are always a mixture of pollen and nectar that can range from a liquid to nearly dry friable masses of pollen. In a few cases, they may also contain floral oils or glandular substances added by the females (Stephn et al., 1969; Neff, 2008). Provisions in *Osmia* spp. are quite dry with about 10–15 % nectar (Ladurner et al., 1999), whereas the provision in *Megachile rotundata* is more liquid (nectar–pollen ratio 2:1) (Cane et al., 2011).

Solitary bees play important roles in ecological systems, particularly in the pollination of crops and wild plants. However, the recent increases in the loss of honey bee colonies and the decline of native

bees in agricultural ecosystems have accelerated the necessity to manage bees for crop pollination (Pitts-Singer and James, 2008). Despite their effectiveness as pollinators, of all the bee species described worldwide, only a handful are managed commercially as crop pollinators, and the biology is well known only for these species.

Life history of *Nomia melanderi*

The alkali bee, *Nomia melanderi* (Hymenoptera Halictidae), is native to west regions of the USA, and by the 1950s this species was the first solitary bee commercially managed for alfalfa (*Medicago sativa*) pollination. These solitary bees are strongly gregarious in their nesting habits, excavating nests in soil in close proximity to each other. In natural conditions it is possible to find large concentrations of nests, up to 4.9 million/ha, and, in artificial nest sites, population levels can reach 24.7 million nests/ha (Pitts-Singer, 2008). The nest tunnel consists of a vertical shaft terminating in a lateral tunnel with nest cells extending downward from it. Each cell is provisioned with nectar and pollen and sealed with a polished soil cap. Only one egg is laid on each provision.

The alkali bee usually emerges as an adult in early July and lives for about 1 month, during which time the nesting female lays up to 20 or 25 eggs. The eggs develop into prepupae (postfeeding fifth instars) in about 15 days (in late July or August) and enter a period of dormancy until June of the following year. The pupal period is relatively short (10 to 15 days at environmental temperature), and adults spend a few days in their cells until their emergence from cocoons. Only one generation per year occurs in most of the north-western USA but in southern California, or in artificial conditions, the alkali bee experiences multiple (up to four) generations. Under such conditions, each generation is expressed over a 30-day period (Stephen, 1965; Johansen and Mayer, 1983).

Life history of *Megachile rotundata*

The alfalfa leaf-cutting bee, *Megachile rotundata* (Hymenoptera Megachilidae), is a gregarious, cavity-nesting, leaf-cutting bee native to south-western Asia and south-eastern Europe. This species was introduced to North America in the 1940s and, currently is considered the most important pollinator for alfalfa (*Medicago sativa*) in central Canada and in the north-western USA (Pitts-Singer and Cane, 2011). After emerging and mating during June and July, depending on the latitude, the alfalfa leaf-cutting bees start building their nests in pre-existing cavities. Nesting continues for 4–6 weeks and nests consist of a linear series of cells constructed from cut-leaf pieces. Each cell requires 14–15 leaf pieces and is provisioned with a mass of pollen and nectar, on top of which an egg is deposited. Completed nests are sealed with cut-leaf plugs. A females spends from 5 to 6 h per day foraging, returning from flowers with both dry pollen in her scopa and nectar in her crop (Klostermeyer and Gerber, 1969; Maeta and Adachi, 2005). By late summer, fifth-instar leaf-cutting bees complete consumption of the pollen–nectar provision, defecate, and spin a cocoon with silk-like strands. In this stage (prepupa), most bees in a given population enter diapause, which lasts through the winter until next year. Diapause terminates with the warming conditions of late spring or early summer, when bees complete their development (Kemp and Bosch, 2000). However, at most latitudes in North America, a small proportion of bees in a given population will avert the late summer prepupal diapause and complete development during the current year (Richards, 1984; Kemp and Bosch, 2000).

Life history of *Osmia* spp.

The genus *Osmia* comprises more than 300 species, mostly in the Holartic (Michener, 2000), and the majority of these species nest in pre-established cavities in which females build series of cells separated by mud or masticated leaf partitions. All species of subgenus *O. (Osmia)* overwinter as adults in their natal cells, are univoltine and fly very early in the year. In part for this reason, several *O. (Osmia)* species have been developed in different parts of the world to pollinate spring-blooming crops (Bosch et al., 2008). In particular, three *Osmia* species, *O. cornifrons*, *O. lignaria* and *O. cornuta*, have been developed as fruit tree pollinators in Japan, the USA and Europe, respectively.

The European mason bee, *Osmia cornuta*, emerges as an adult in March–April and flies for about 3 weeks. The larval feeding period ranges from 25 to 40 days and the prepupal period lasts for about 1–2 months (depending on the geographical origin area). The pupal period lasts for 20–30 days, and the adult ecloses within the cocoon in late summer (Krunic and Stanisavljevic, 2006; Bosch et al., 2008). Adults remain inside their cocoons in a dormant stage throughout the winter period and they emerge in the spring as temperatures increase (Bosch and Kemp, 2004; Sgolastra et al., 2010a).

D. SOURCES OF EXPOSURES FOR HONEY BEES

Sugar intake from nectar from plants

Nectar, pollen and water foragers: while water and pollen foragers consume all the sugar they require for their flight at the hive before leaving, nectar foragers need as much sugar to leave the hive (i.e. nectar/honey stored in the hive; i.e. 2.A.a) as to return to the hive (i.e. nectar on flowers) in the case of long-distance/duration flights.

Wax-producing bees, nurse/brood-attending bees, drones, queens and larvae: with the exception of nectar foragers, all categories of bees do not consume nectar directly on flowers but in the hive. However, for winter bees (1D), towards the end of winter, if weather conditions are good, honey bees will collect nectar and start rearing brood. During that time, winter bees will be exposed to the same amounts of nectar as described for nectar foragers from plants (1.A.a), i.e. 32–128 mg/day.

Sugar intake from nectar/honey stored in the hive

Nectar and pollen foragers: a forager needs 8–12 mg/h of sugar to fly. While a nectar forager achieves on average 10 trips per day, each of about 30–80 minutes, a pollen forager achieves on average 10 trips per day of about 10 minutes each. Given that foragers spend 80 % of their time flying (and 20 % foraging, but we cannot estimate the energy cost for this activity), nectar foragers need a minimum of 32–128 and pollen foragers 10–16 mg of sugar per day (see Rortais et al., 2005). However, the number of trips achieved by bees is highly variable, depending on the type of flowers visited (see Table 3.10.1, p. 173, in Winston, 1987) and weather conditions.

Water foragers: given that foragers consume 8–12 mg/h of sugar during flying and that water foragers make five trips per hour and a total of 46 trips per day, water foragers need about 72–110 mg of sugar per day (see Seeley, 1995).

Wax-producing bees: over the period of maximum wax production (lasting a minimum of 6 days), a bee produces 3 mg of wax per day, requiring 18 mg of sugar per day (Rortais et al., 2005).

Nurse/brood-attending bees: from April to October, under temperate weather conditions, temperatures are about 15–20 °C. In these conditions, brood attending bees consume 34 mg (at 20 °C) to 50 mg (at 15 °C) of sugar per day to maintain the brood temperature at 34 °C (Rortais et al., 2005).

Winter bees: in temperate regions, during winter, bees consume 8.8 mg of sugar per day to maintain the nest temperature at 5–8 °C in the periphery and 15–20 °C in the centre (Rortais et al., 2005). Towards the end of winter, if weather conditions are good, honey bees will collect food (nectar and pollen) and rear brood. Therefore, bees will consume similar amounts of honey as described in 1.A.a, 2.A.a and 2C, i.e. 32–128 mg/day to collect nectar or 34–50 mg/day to rear brood. However, the energy required to maintain the brood at 34 °C (during spring/summer) and to maintain the nest at 15–20 °C in the centre (during winter) depends on climatic conditions related to the geographic location.

Drones: drones make 3–5 mating flights per day, lasting 30–60 minutes each, and drones need 14–18 mg of sugar per hour of flight activity during the mating period (Winston, 1987; Seeley, 2002). Therefore, drones will need 21–90 mg of sugar per day to achieve these flights.

Queens: queens make about 2–3 mating flights per day, lasting 90 minutes each. Assuming that drones and queens have the same metabolic rate (because they have about the same body mass), then queens would require the same amount of sugar as drones, i.e. 14–18 mg of sugar per hour of flight (Winston, 1987; Seeley, 2002). Therefore, queens will need 42–81 mg of sugar per day of flight during the mating season.

Larvae: based on the length of the development of larvae (5 days for worker larvae and 6.5 days for drone larvae) and the body weight increase, Rortais et al. (2005) estimated the sugar consumption of worker and drone larvae during their whole development at 59.4 mg and 98.2 mg, respectively.

Pollen intake and contact with pollen from flowers

Nectar foragers: a nectar forager can be in contact with pollen when collecting nectar but no quantification could be found.

Pollen foragers: a pollen forager carries about 15 mg of pollen per trip. If we assume that a pollen forager makes on average 10 trips/day, it will be in contact with about 150 mg of pollen per day (Winston, 1987). However, the number of trips achieved by bees is highly variable, depending on the type of flowers visited (see Table 10.1, p. 173, in Winston 1987), but also on weather conditions.

Water foragers: it is assumed that water foragers will not be in contact with flowers and pollen; or if they do it is assumed that it will be negligible.

Hive bees: all categories of honey bees are in contact with wax and propolis in the hive. Negligible amounts of pollen are present in wax (see Chauvin, 1968, volume 3, p. 47). In addition, bees which manipulate pollen in the hive (for storing and preparation of food larvae) are in contact with the following amounts of pollen:

- **Nurse/brood-attending bees:** bees which store pollen in the hive are in contact with similar (or most probably higher) amounts of pollen consumed by nurse bees as defined in 4.C (i.e. consumption of pollen from bee bread by nurse/brood-attending bees), i.e. ≥ 6.5–12 mg/day.

- **Winter bees:** as explained in 2.D (i.e. consumption of honey by winter bees), towards the end of winter, if weather conditions are good, honey bees will collect and store pollen and be in contact with similar amounts of pollen as defined in

    - (i) 3.A.b (i.e. consumption of pollen from flowers by pollen foragers), i.e. 150 mg/day, or

    - (ii) 4.C (i.e. consumption of pollen from bee bread by nurse/brood-attending bees), i.e. 6.5–12 mg/day.

Pollen intake from bee bread

Foragers, wax-producing bees, queens and queen larvae: these categories of bees do not consume pollen. The food of queens is made of royal jelly which does not contain pollen – see Planta (1888) and Haydak (1943) described in Rortais et al. (2005) and Smith (1959).

Nurse/brood-attending bees: nurses consume pollen during the first 8–10 days of their life to develop their hypopharyngeal and mandibular glands and to produce the larval food, but they can consume pollen until the age of 18 days or even over longer periods of time (see footnote c in Table 3.14). It is estimated that the amount of pollen consumed by nurses is 6.5–12 mg/day (Rortais et al., 2005).

Winter bees: as explained in 2.D (i.e. consumption of honey by winter bees) and 3.D (i.e. consumption of pollen from flowers by winter bees towards the end of winter), if weather conditions are good, honey bees will rear brood. In such a situation, the consumption of bee bread by nurse/brood-attending bees is unknown, but it is assumed that the same amount of pollen as defined in 4.C (i.e. consumption of pollen from bee bread by nurse/brood attending bees) would be required to rear a new brood, i.e. 6.5–12 mg/day.

Drones: drones eat less pollen, and over a shorter period (the first days after emergence), than workers (Hrassnigg et al., 2005). Based on the fact that the amount of pollen found in drones is 2–3 % of the amount found in workers' gastrointestinal tract, it is assumed that the consumption of pollen by drones is negligible (i.e. a maximum of 3 % of 12 mg per day during the first days after emergence, i.e. < 0.36 mg/day).

Drone and worker larvae: in experimental conditions, worker larvae feed on 1.5–2 mg of pollen and drone larvae have 36 % more pollen in their gut but the same assimilation rate (Babendreier et al., 2004). Therefore, drone larvae can consume 2.04–2.72 mg of pollen during their development.

Sugar intake from honey dew collected on plants

Honey dew foragers: foragers, whether collecting nectar or honey dew, need the same amount of sugar for flying back to the hive. We can assume that the amount of sugar required to collect honey dew is the same as the amount of sugar required to collect nectar, i.e. 32–128 mg/day (see 1.A.a or 2.A.a, i.e. consumption of nectar from flowers or stored in-hive by nectar foragers).

Hive bees: with the exception of honey dew foragers, all categories of bees do not consume honey dew directly on flowers but in the hive. In the southern EU, weather conditions are favourable to *Aphis* populations until late autumn. Under these conditions, honey bees may collect and store honey dew in the hive which will be later used by winter bees as defined in 1.A.a (i.e. consumption of nectar from flowers by nectar foragers), i.e. 32–128 mg/day.

Sugar intake from honey dew stored in the hive

If we assume that all the sugar consumed by the different categories of bees comes from honey dew (rather than nectar), the required amounts of sugar coming from honey dew will be the same as the required amounts of sugar coming from nectar as defined in 2.A–G.

Honey bees are known to collect honey dew primarily from tree-feeding aphid species such as *Sternorrhyncha* but they have also been regularly shown to exploit honey dew from aphid-infested cereal and other crops, e.g. potatoes (Maurizio, 1985). Other bee species have also been reported to collect honey dew, such as *Osmia* (Konrad et al., 2009b) and bumble bees (Bishop, 1994).

The sugar content of aphid honey dew is highly influenced by both the species of aphid and the host plant (Fischer and Shingelton, 2001). Hogervorst et al. (2007) and Wykes (1953) reviewed the sugar content of aphid honey dews from different aphid species and host plants. Aphids not only excrete the sugars taken up from the plant phloem but also synthesise specific sugars, such as the trisaccharide melezitose, to attract ants. Species-specific differences are demonstrable, with aphids on some plants excreting up to 30–70 % melezitose whilst other species of aphid not tended by ants excrete no melezitose (Fischer and Shingelton, 2001).
Leroy et al. (2011) reported that plant-derived phloem sugars account for 67–89 % of the sugar content of honey dews. Owing to the small volume of exudates available Fischer and Shingleton (2001) could not ascertain the actual content of sugars in aphid honey dew but did report that there were wide differences in the sugar composition of excreta of three species of aphids feeding on two species of poplar and composition depended on whether ants were present.
Hogervorst et al. (2007) identified that although honey dew may be a food source (e.g. for parasitoids) it can be far inferior to nectar based on sugar composition, amino acid composition and the presence of

plant secondary chemicals. However, honey dew on crops may be a significant percentage of the sugar flow into colonies at times of limited, or less attractive, alternative forage.

Water intake from water surface, puddles, leave axils and droplets on leaves

Water foragers: estimates give an average of 46 trips per day for water foragers (Seeley, 1995). If bees carry 30 µl and up to a maximum of 58 µl of water in their crop (Visscher et al., 1996), they will carry a total of 1.4–2.7 ml of water per day.

Hive bees: the water brought back by foragers is needed to maintain an osmotic balance in the adult bees, to prepare liquid food for the brood and to cool the hive on hot days. At the colony level, it is estimated that 20–25 litres is consumed annually (Weipple, 1928; Seeley, 1995). However, this amount is highly variable, depending on weather conditions. For example, for the entire spring/summer season, Michailoff (1961) found that a colony can use up to 42 litres and, according to Farrar (1973), during the warm season on hot days, a colony can consume up to 20 L/week (or 2.9 L/day).

Contact with propolis

Foragers: foragers can carry 30 mg of propolis per trip (Seeley, 1995). The daily number of trips achieved by propolis foragers is unknown. If we assume that within a day a forager can achieve a minimum of one trip to collect propolis and a maximum of 10 trips, as described for pollen and nectar foragers, the possible amount of propolis in contact with the forager will be 30–300 mg per day. However, the number of trips achieved by bees is highly variable, depending on the type of flowers visited (see Table 10.1, p. 173, in Winston (1987)), but also depending on weather conditions.

Hive bees: we can assume that all categories of honey bees (except larvae) may be in contact with propolis in the hive. Negligible amounts of pollen are present in propolis (see Chauvin, 1968, volume 3, p. 47). Therefore, we cannot exclude an exposure to pollen via propolis, but it is difficult to estimate it.

E. SOURCES OF EXPOSURE TO BUMBLE BEES

Adults

- Worker pollen intake

Tasei and Aupinel (2008) reported that the pollen intake of three workers during a 5-day nest construction/egg-laying period (this includes the processing/deposition of pollen in the nest material) was a mean of 0.38 mg over 5 days (0.076 g/day) or 0.38/5 days × 3 workers = 0.03 g/day/worker for nest construction. This agrees with the data reported by Tasei et al. (2000) of 26.6–30.3 mg/day/worker.

- Adult sugar intake

Tasei et al. (2000) reported that for microcolonies the mean consumption of sugar syrup (37 % sucrose; 38 % fructose/dextrose) per day was 0.179–199 mg/day/worker (134–149 mg sugar/day/worker). Tasei et al. (1994) reported that, over a 4-week period in which workers were allowed access to untreated syrup, the mean intake per day was 0.208 g of sugar solution per worker; based on 35 % sugar content, the mean sugar intake was 73 mg sugar/day.

- Forager nectar intake

There are very limited data on the uptake of nectar by foragers. Data provided by Prys-Jones and Corbet (1991) and Crane (1990) suggests that the intake of nectar at plants by *B. pascuorum* workers

(weighing 74–165 mg) is 36.0–65.1 µl per visit and by *B. terrestris* workers (weighing 109–300 mg) is 41.1–111.9 µl.

Larvae

Pereboom (2000) assessed the relationship between the composition of larval food and the development of female castes in bumble bees (*B. terrestris*). They reported that, unlike honey bees, individual bumble bee workers do not change the composition of the food they provide to the brood in relation to development of either queens or workers. Pendrel and Plowright (1981) showed that the size of adult *B. terrestris* and *B. terricola* workers is correlated with larval intake of pollen and that feeding rates are correlated with pollen availability. Feeding in *Bombus* colonies is far less well regulated than in honey bee colonies and related to overall larval biomass rather than feeding of individual larvae, with individual larvae fed between one and three times per hour over a 24-hour period. Brian (1952) reported that the larvae of *B. agrorum* usually receive three or four feedings per hour. The construction of the bumble bee nest and the fact that eggs are laid in clusters and only move apart into separate cells as they develop makes estimating larval consumption of food difficult.

- Larvae pollen intake

Retrieval of the food supplied to worker, queen and male larvae (Pereboom, 2000) showed that the average amount was 0.88 µl, with a only slight correlation between the size of the food sample and the age of the larva, with pollen constituting 34 % of the total sample (0.03 ng approximately). If larvae are fed three times per hour and 24 hours per day, this equates to 72 feeds per day of 0.88 µl or 64 µl/day/larva, comprising 22 mg pollen/day. Bumble bee workers do not feed glandular secretions to larvae, unlike honey bees, and the pollen is fed relatively unprocessed.

Pereboom (2000) reported that provisioning bees drink sucrose prior to consuming pollen and the amount of pollen ingested depends on the protein content of the pollen, and this is supported by Tasei and Aupinel (2008). During the period up until pupation (14 days), Tasei and Aupinel (2008) reported that a microcolony containing a mean of 4.8 larvae consumed a mean of 2.63 g of *Castanea* pollen (this pollen produced the greatest mean weight of larvae in a microcolony) over 14 days or 0.188 g/day. This intake includes the consumption required for the continued colony construction by the workers (0.076 g/day) so the larvae alone consumed 0.188 – 0.076 = 0.112 g/4.8 larvae = 23 mg/day/larva. Although these are data for small numbers of workers and drone larvae (laying workers produce haploid eggs which develop into drones), they can be used to estimate intake rates by different castes of bumble bee larvae (workers/queens/drones) as Pereboom (2000) suggested that intake varies little between the castes; rather, it is the length of the feeding period (larval development period) that is important.

- Larvae sugar intake

Assuming that the balance of the 64 µl/day fed to bumble bee larvae by workers (34 % is pollen) (Pereboom, 2000) is sucrose (50 % w/v) (since workers consume sucrose prior to pollen for feeding the larvae) then this is equivalent to 47.5 mg sucrose/day/larva or 23.8 mg sugar/day.

F. SOURCES OF EXPOSURE TO SOLITARY BEES

Osmia cornuta populations nest for about 50 days in spring (from March to May depending on the population origin), during which time exposure of adults to pesticide is possible (Bosch and Vicens, 2005, 2006). During the nesting period each female visits about 10 000–25 000 flowers in ~20 days (Bosch, 1994) and consumes about 360–1 540 mg of sugar. In fact, assuming that *Osmia* needs 8–12 mg/h of sugar to fly (as honey bee forager), a nesting female achieves 12 foraging trips/day of 0.16–0.5 h each, plus five mud trips per day, of 0.08 h each (Bosch, 1994; Bosch and Vicens, 2005). Excluding the energy spent during pollen and mud deposition in the nest, a nesting female needs at least 18–77 mg of sugar per day. Like other solitary bees, *Osmia* females need to eat pollen to

complete ovary maturation during the pre-nesting period, but we cannot estimate the amount of pollen consumed (Richards, 1994; Sgolastra, 2007). Instead, it is possible to estimate the daily quantity of pollen with which bees come in contact by considering the dry weight of pollen loads carried to the nest by females in each foraging trip (10.7 mg) and the mean daily number of foraging trips (12) (Bosch and Vicens, 2005). The amount of pollen which each bee comes in contact with during the nesting period is estimated by the number of nests produced per female (2–4) and the quantity of pollen transported in each nest (1.2 g pollen dry weight/nest) (Bosch and Vicens, 2005, 2006). Adult bees can be exposed to pesticide in soil; in fact, during the nesting period, each female collects about 1.1 g of mud (dry weight) per nest and 2.2–4.4 g of mud during all the nesting period (Bosch and Vicens, 2005). The mean dry weight of mud loads carried to the nest by nesting females was 16.5 mg (Bosch and Vicens, 2005); thus, an *O. cornuta* female carries to the nest about 82.5 mg of mud per day. Individual females are active for 20–25 days (including the pre-nesting period) and build 0.5–1.5 cells per day under field conditions (Bosch, 1994; Bosch and Vicens, 2005, 2006). In orchard environments, each female may build a total of 8–18 cells with a sex ratio (F:M) of 0.65 (Bosch, 1994; Bosch and Vicens, 2005, 2006). The feeding period of larvae lasts about 1 month (Krunic and Stanisavljevic, 2006; Bosch et al., 2008), and during this period male and female bees consume a mean of 318 and 542 mg of mass provision, respectively (Bosch and Vicens, 2002). Provisions stored by bees are always a mixture of pollen and nectar, but provisions in *Osmia* are quite dry, containing only 10–15 % nectar (Ladurner et al., 1999). Compared with honey bee larva, the larvae of solitary bees are much more exposed to residues in pollen.

Adult alfalfa leaf-cutting bees (*Megachile rotundata*) naturally emerge during the summer and populations nest for about 2 months. Females mate soon after emergence and then consume nectar and pollen as their first eggs mature (Richards, 1994). They nest in existing holes above ground, and each nest consist of a linear series of cells delimited by cut-leaf partition (on average 14 or 15 leaf pieces per cell) (Pitts-Singer and Cane, 2011). Bees (average weight 35 mg) are able to carry leaf piece loads of 17 % of their body weight, and during the nesting period they can collect up to 4.9 g of leaves (Klostermeyer et al., 1973). Nesting females sometimes lay two eggs per day, and each female completes, on average, 30–50 cells with eggs over the lifespan (from 4 to 8 weeks) (Pitts-Singer, 2008; Pitts-Singer and Cane, 2011). The pollen load transported by bees in each foraging flight is about 1.6 mg (4.5 % of body weight) (Klostermeyer et al., 1973; Neff, 2008); given that a total of 15–30 foraging trips are required for each cell (Mader et al., 2010), a nesting female carries 48–96 mg of pollen per day and 1.3–5.4 g of pollen during the nesting period. Considering that a female spends 5–6 hours per day foraging (Klostermeyer and Gerber, 1969; Maeta and Adachi, 2005), the sugar consumed per day ranges from 44 to 66 mg, and total consumption during the whole nesting period ranges from 1 848 to 2 772 mg. These values are calculated assuming an energetic cost of 8–12 mg of sugar per hour per bee, as for honey bee foragers. Although the energetic cost can be affected by body size, physiology and climatic conditions, the energetic cost calculated for *M. rotundata* is a little higher than values estimated for two other species of *Megachile* (Abrol, 1986). The sex ratio of males–females ranges from 2:1 in natural populations to 1:1 in commercial populations reared in the USA (Pitts-Singer, 2008).

Like other solitary bees, *M. rotundata* larvae are much more exposed to residues in pollen because the feeding is different (unprocessed pollen is fed to larvae) and the amount of pollen exposure is higher than honey bee larvae. In fact, the provision weighs about 90–94 mg, and consists of 33–36 % pollen and 64–67 % nectar by weight (Cane et al., 2011). It contains about 1.3 million pollen grains and male provision weighs 17 % less than female provision (Pitts-Singer and Cane, 2011). The larva feeding period lasts about 10 days (Kemp and Bosch, 2000).

G. RESIDUES IN NECTAR AND POLLEN FOLLOWING SPRAY APPLICATIONS AND SYSTEMIC SEED TREATMENTS

Spray applications on flowering crops or honey dew cause contamination of nectar and pollen. Residues may also be translocated to nectar and pollen from SSST. Residue data are available for only a limited number of substances in nectar, pollen and honey. The concentration in nectar and pollen can be used to predict exposure of both foraging bees and bees other castes in the hive, including larvae.

Data on residues in nectar and pollen following spray applications and systemic seed treatments were obtained from published literature, the DARs and from industry studies.
For the different seed treatments, the maximum residues measured in the corresponding studies and the application rate of the a.s. per hectare (tables G1-G11) a theoretical calculation was made which residues could be expected if the application rate of the seed treatment was 1 kg a.s. per hectare. If the residue detected in the study was between LOD (e.g. 0.3) and LOQ (e.g. 1) , we made the worst case assumption that the residue was equal to the LOQ (1 in this case) for calculation. When several measurements of residues for the same matrix, e.g. pollen were available in a study, only the highest value was used for the calculation.
Further information on residue content found in homing foragers several days after application is provided in Figure G1.

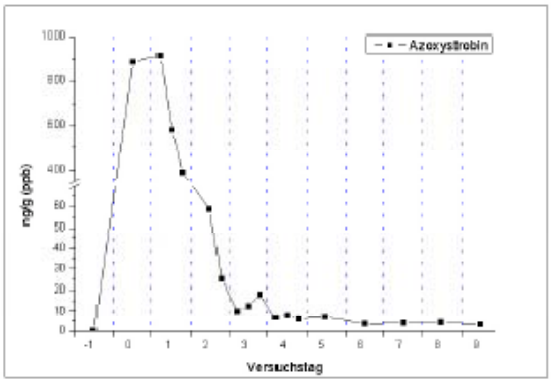


Abbildung 40: Verlauf der Konzentration von Azoxystrobin im Nektar von Volk 1 am Ihinger Hof

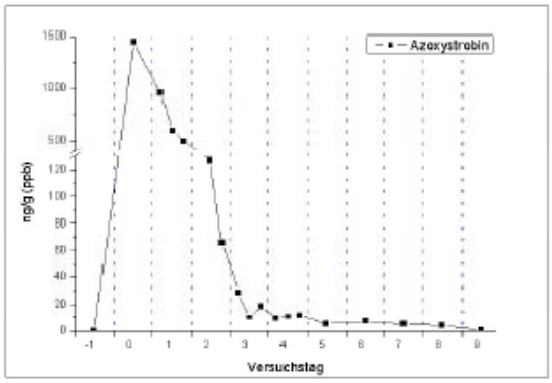


Abbildung 41: Verlauf der Konzentration von Azoxystrobin im Nektar von Volk 2 am Ihinger Hof

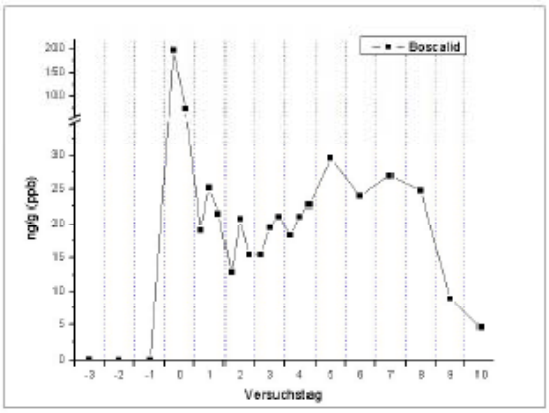


Abbildung 42: Verlauf der Konzentration von Boscalid im Nektar von Volk 3 am Heidfeldhof

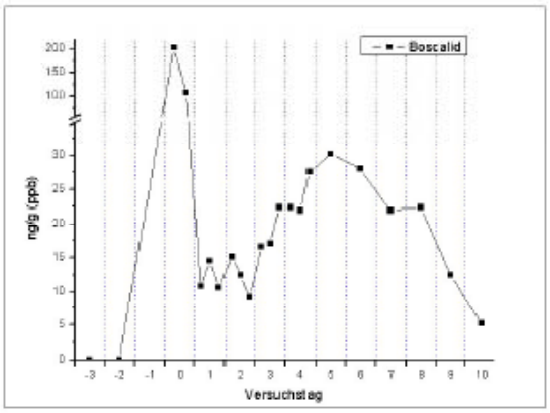


Abbildung 43: Verlauf der Konzentration von Boscalid im Nektar von Volk 4 am Heidfeldhof

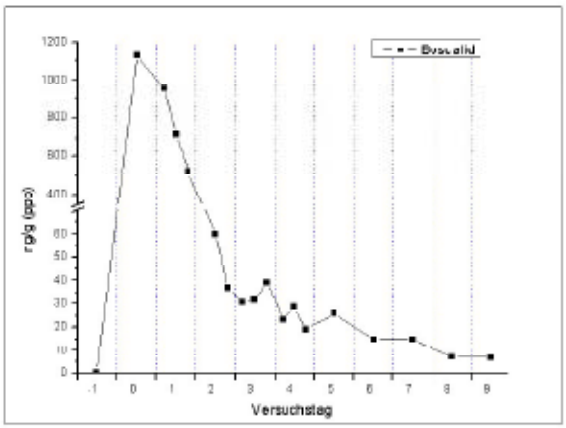


Abbildung 44: Verlauf der Konzentration von Boscalid im Nektar von Volk 1 am Ihinger Hof

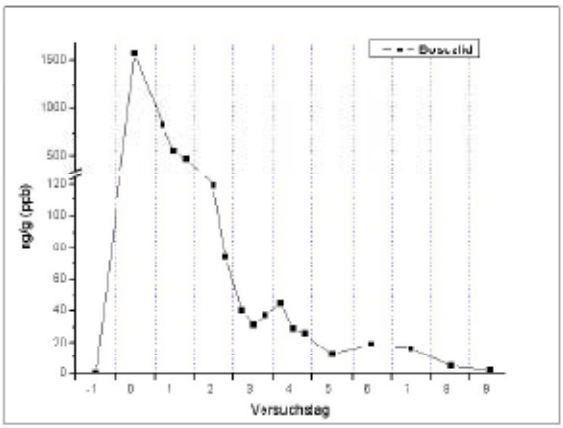


Abbildung 45: Verlauf der Konzentration von Boscalid im Nektar von Volk 2 am Ihinger Hof

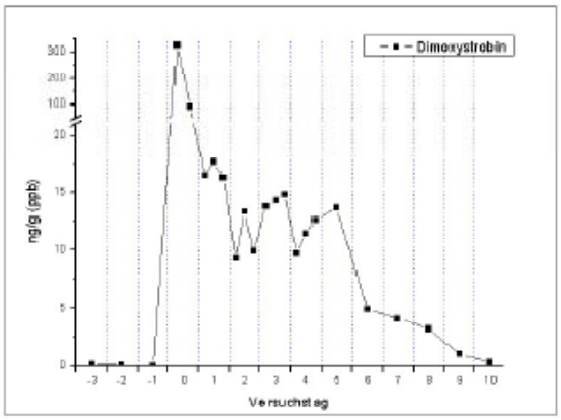


Abbildung 46: Verlauf der Konzentration von Dimoxystrobin im Nektar von Volk 3 am Heidfeldhof

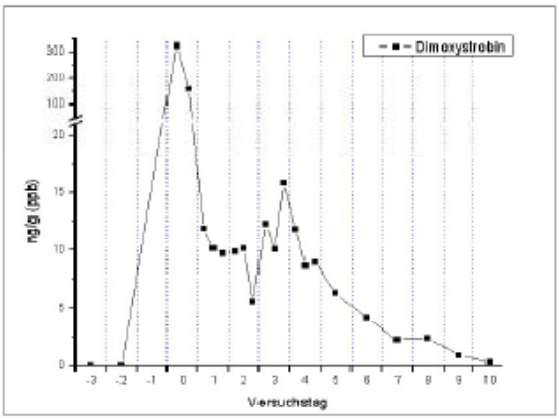


Abbildung 47: Verlauf der Konzentration von Dimoxystrobin im Nektar von Volk 4 am Heidfeldhof

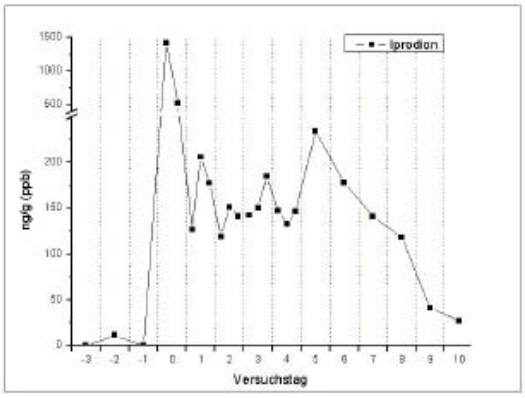


Abbildung 48: Verlauf der Konzentration von Iprodion im Nektar von Volk 3 am Heidfeldhof

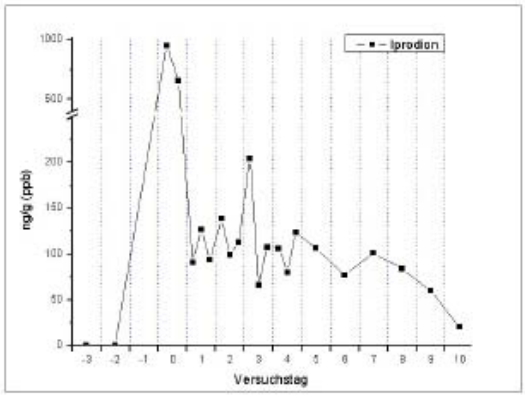


Abbildung 49: Verlauf der Konzentration von Iprodion im Nektar von Volk 4 am Heidfeldhof

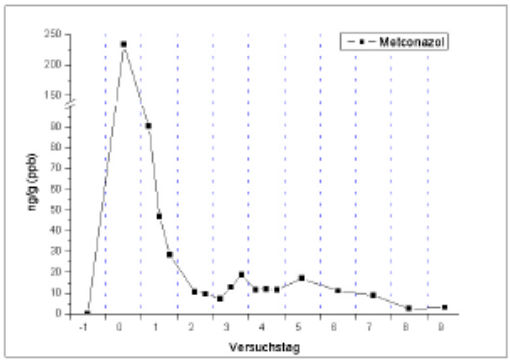


Abbildung 50: Verlauf der Konzentration von Metconazol im Nektar von Volk 1 am Ihinger Hof

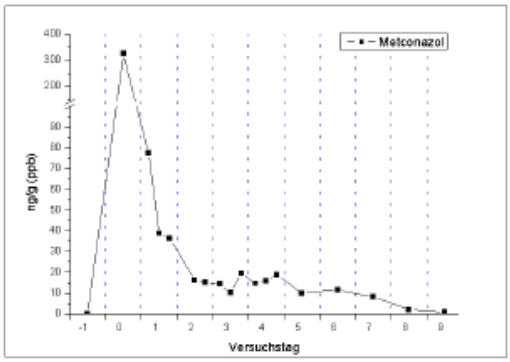


Abbildung 51: Verlauf der Konzentration von Metconazol im Nektar von Volk 2 am Ihinger Hof

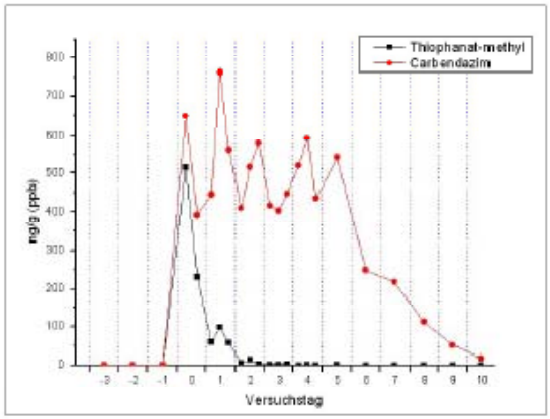


Abbildung 52: Verlauf der Konzentration von Thiophanat-methyl und Carbendazim im Nektar von Volk 3 am Heidfeldhof

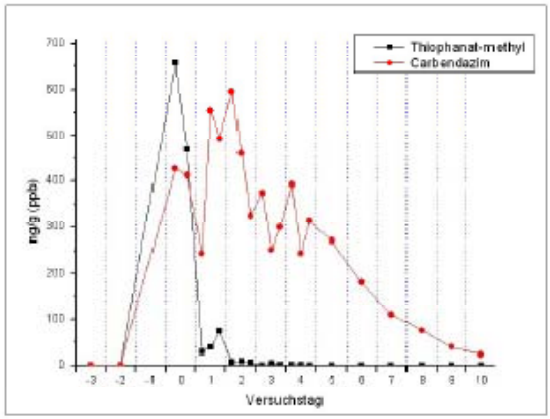


Abbildung 53: Verlauf der Konzentration von Thiophanat-methyl und Carbendazim im Nektar von Volk 4 am Heidfeldhof

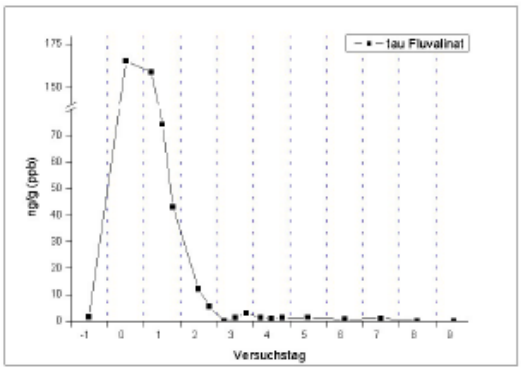


Abbildung 54: Verlauf der Konzentration von tau-Fluvalinat im Nektar von Volk 1 am Ihinger Hof

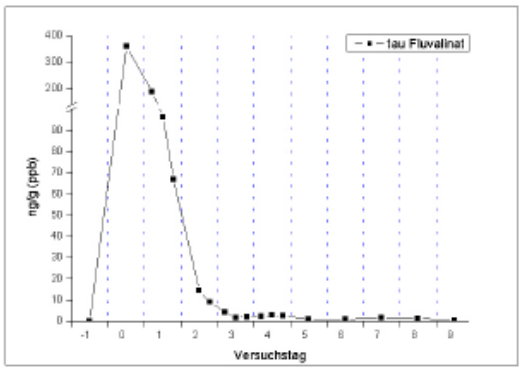


Abbildung 55: Verlauf der Konzentration von tau-Fluvalinat im Nektar von Volk 2 am Ihinger Hof

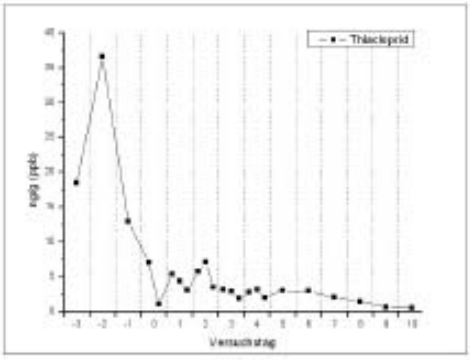


Abbildung 56: Verlauf der Konzentration von Thiacloprid im Nektar von Volk 3 am Heidfeldhof

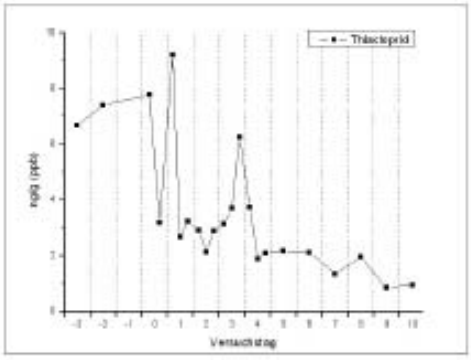


Abbildung 57: Verlauf der Konzentration von Thiacloprid im Nektar von Volk 4 am Heidfeldhof

Figure G1: Course of the concentration of the active substance in nectar of homing foragers of individual colonies (e.g. colony 3) following application in flowering winter oilseed rape during daily bee flight at Heidtfeldhof (Stuttgart-Hohenheim, Germany) (days before/after application) (Schatz and Wallner, 2009).

More data on residues in honey from different monitoring studies and literature are presented in Tables G1–5 from different studies and may be used for estimates on long-term exposure. In the study from Schatz and Wallner (2009) (see Figure G1 and Table G7), residues in nectar from forager bees and for some substances in ripened honey from the same trial were analysed, indicating generally lower residue values in honey. This conclusion may also be derived from studies with colonies fed a solution containing different concentrations of the tested substance. Also, decrease patterns (which are always influenced by individual substance properties) follow the same mechanisms (Thompson et al., 2005; Adams et al., 2007, 2008, 2009). In a review (Bogdanov, 2006) of contaminants of bee products including honey, it was concluded that the low concentration of pesticides in honey is due to a filtering effect of bees. Indeed, bees decrease initially high pesticide nectar concentrations, so that the final concentration in honey is much lower, usually by a factor of about 1000 (Schur and Wallner, 1998, 2000). Also, several of the new and currently used pesticides are unstable and degrade quickly after use (Bogdanov, 2006).

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 slug
Mercaptodimethur sulfon	ND	5.0/10.0	A	ND	3.5/10.0	A				11.5	5.0/10.0	A	Anti slug
Mercaptodimethur sulfoxide	ND	5.0/10.0	A	ND	3.5/10.0	A				ND	5.6/10.0	A	Anti slug
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

Table G2: Residue table: data from the DAR on clothianidin (Belgium, 2003) from seed treatment

No	Crop	Tested dose mg/seed	Tested dose g/ha	LOD/LOQ (µg/kg)	Compound	Nectar (µg/kg)	Honey (µg/kg)	Pollen (µg/kg)	Honey bees (µg/kg)	Conditions	Country /year	Reference as cited in Belgium (2003)
1	Summer rape		27[f] (0.045 l)	0.3/1	Clothianidin (TI-435)	2.8–3.0				Tent in field	D/2000	Maus and Schöning (2001b)
				0.3/1	Metabolite (TZMU)	ND/0.3<R<1						
				0.3/1	Metabolite (TZNG)	ND/ND						
2	Summer rape		27[f] (0.045 l)	0.3/1	Clothianidin (TI-435)	1 5.4[a]	ND[c]	1.9 2.5*		Tent in field	D/2000	Maus and Schöning (2001c)
				0.3/1	Metabolite (TZMU)	ND/0.3<R<1	ND[c]	ND				
				0.3/1	Metabolite (TZNG)	ND/ND	ND[c]	ND				
7	Winter rape	0.41	48.9	0.3/1	Clothianidin (TI-435)	0.3<R<1/1	0.3<R<1/1[c]	0.3<R<1[b]		Tent in field	D/2001	Maus (2002a)
					Metabolite (TZMU)	ND	ND[c]	ND[b]				
					Metabolite (TZNG)	ND	ND[c]	ND[b]				

9	Summer rape		49.8[f] (0.083 l)		Clothianidin (TI-435)	8.6[d] 1.2–7.2[a]		4.1[e]	1.4	Tent in field	S/1998	Schmuck and Schöning (2000a)
10	Summer rape		49.8[f] (0.083 l)		Clothianidin (TI-435)	sample too little	sample lost	3.3[e] sample too little	sample too little	Tent in field	GB/2000	Schmuck and Schöning (2000b)
11	Summer rape		49.8[f] (0.083 l)		Clothianidin (TI-435)	0.3 <R<1[d] 0.3<R<1[a]		1.7[d] 0.3<R<1[e]	ND	Tent in field	F/1998	Schmuck and Schöning (2000c)
12a	Spring canola		42		Clothianidin (TI-435)	0.9–3.7		1.5–3		Field	Ca/2000	Scott-Dupree and Spivak (2001)
12b	Spring canola		30		Clothianidin (TI-435)	1.0–1.1		2.3–2.8		Field	USA/2000	Scott-Dupree and Spivak (2001)
3	Sunflowers	0.29	25.4	0.3/1	Clothianidin (TI-435)		ND[c]	2.4/3.1*[a] 1.2/1.3*[b]		Tent in field	D/2000	Schmuck and Schöning (2001d)
				0.3/1	Metabolite (TZMU)		ND[c]	0.3 < R< 1[a] ND[b]/ND*[b]				
				0.3/1	Metabolite		ND[c]	0.3<R<1[a]/ND				

					(TZNG)			ND[b]/ND*[b]				
4	Sunflowers	0.29	25.4	0.3/1	Clothianidin (TI-435)		ND[c]	2.3/2.4*[a] 2.6/2.9*[b]		Tent in field	D/2000	Maus and Schöning (2001e)
				0.3/1	Metabolite (TZMU)		ND[c]	ND/ND*[a] 0.3<R<1/ND*[b]				
				0.3/1	Metabolite (TZNG)		ND[c]	ND/ND*[a] 0.3<R<1/ND*[b]				
8	Maize		53.8		Clothianidin (TI-435)			5.4/3.3* 6.2		Tent in field	D/?	Maus and Schöning (2001f, 2001g)
					Metabolite (TZMU)			ND/ND* ND				
					Metabolite (TZNG)			ND/ND* 0.3<R<1				
8b	Maize		53.8		Clothianidin (TI-435)			2.4/2.9 2.1		Tent in field	D/?	Maus (2002b, 2002c)
					Metabolite (TZMU)			ND ND				
					Metabolite (TZNG)			ND ND				

data missing
or
incomplete
in the DAR

R: concentration of the residue

ND: not detected

*Repetition of the first analysis

a: from the flower (for nectar = capillary)

b: from the comb

c: nectar from the honeycomb

d: sampled by bes

e: blossom

f: estimate (not in the DAR)

Table G3: Residue table: data from the DAR on thiamethoxam (Spain, 2001) from seed treatment

No	Crop	Tested dose		LOD/LOQ (µg/kg)	Compound	Flower (heads) (µg/kg)	Pollen (on bees) (µg/kg)	Honey (µg/kg)	Honey stomach (µg/kg)	Pollen (µg/kg)	Conditions	Reference as cited in Spain (2001)
		mg/seed	g/ha									
A	Oilseed rape	0.0189	34	1/?*	Thiamethoxam	1.8					Cruiser WS70	Schur (2001a)
					CGA322704	< 1						
		0.0372	67		Thiamethoxam	3.9					Semi-field (cage)	
					CGA322704	1.3						
		0.0561	101		Thiamethoxam	2.3						
					CGA322704	< 1						
		0.0744	134		Thiamethoxam	13						
					CGA322704	4.2						
		0.1122	202		Thiamethoxam	14						
					CGA322704	4.9						
		0.151	269		Thiamethoxam	27						
					CGA322704	10						
C	Sunflower	0.175	26	1/?*	Thiamethoxam	< 1					Cruiser WS70	Schur (2001b)
					CGA322704	< 1					Semi-field (tunnel)	
D	Sunflower	0.175	26	1/?*	Thiamethoxam	1		<1			Cruiser WS70	Barnavon (1999
					CGA322704	<1		<1			Semi-field (tunnel)	
		0.35	52.5		Thiamethoxam	1		<1				
					CGA322704	<1		<1				

E	Sunflower	0.175	26	1/?*	Thiamethoxam	< 1	< 1	< 1			Cruiser WS70	Barnavon (1999)
					CGA322704	<1	< 1	<1			Semi-field (tunnel)	
F	Oilseed rape	0.016	26	1/?*	Thiamethoxam	3.1–4.2	2.5–4.2	1–<1	1–2.1	2.8	Cruiser WS70	Schuld (2001a)
					CGA322704	<1	< 1	< 1	<1	<1	Field	
G	Oilseed rape	0.017	29	1/?*	Thiamethoxam	<1–4.6	< 1	< 1			Cruiser WS70	Schuld (2001b)
					CGA322704	<1–1	< 1	< 1			Field	
H	Sunflower	0.175	28	1/?*	Thiamethoxam	3	<1–1.1	< 1–1	<1		Cruiser WS70	Balluf (2001)
					CGA322704	<1	<1	< 1	<1		Field	
I	Sunflower	0.25	22	1/?*	Thiamethoxam	<1	<1–3.2	<1			Cruiser WS70	Schur (2001c)
					CGA322704	<1	<	<1			Field	
J	Sunflower	0.28	19	1/?*	Thiamethoxam	2	< 1	<1[b]			Cruiser 350FS	Szentes (2001a)
						1[a]		<1[c]			Field	
					CGA322704	<1	< 1	<1[b]				
						<1[a]		<1[c]				
K	Sunflower	0.28	18	1/?*	Thiamethoxam	1	< 1	< 1[b]			Cruiser 350FS	Szentes (2001b)
						<1[a]		< 1[c]			Field	
					CGA322704	<1	< 1	< 1[b]				

						<1[a]		< 1[c]				
L	Oilseed rape	0.018	18	1/?*	Thiamethoxam	1	<1	<1[b]	<1		Cruiser 350OSR	Schur (2001d)
					CGA322704	<1	<1	<1[b]	<1–1		Field	
M	Oilseed rape	0.016	31	1/?*	Thiamethoxam	3.6		< 1[b]	< 1	<1–3.6	Cruiser 350OSR	Barth (2001)
					CGA322704	<1		< 1[b]	< 1	<1–1	Field	
O	Oilseed rape	0.018	26–27	0.4/?	Thiamethoxam	<0.4–7.5		< 0.1–0.9	<0.02–0.05[d]	<0.2–0.7	Helix	Purdy (2000)
				0.1/?	CGA322704	<0.4–0.9		< 0.1	<0.02–0.05[d]	<0.2–0.24	Field	
				0.2/?								
					a: flowers							
	R: concentration of the residue				b: fresh honey							
					c: fresh nectar							
					d: macerated bees							
	*It should be noted that the value of the LOD is very high, taking into account what we now know about the level of residues in nectar and pollen, and the high toxicity of these compounds.											

Table G4: Residue table: data from the DAR on imidacloprid (Germany, 2005) from seed treatment

No	Crop	Tested dose		LOD/LOQ (µg/kg)	Compound	From flower		From comb/trap/bee			Conditions	Reference as cited in Germany (2005)/location of the study
		mg/seed	g/ha			Nectar (µg/kg)	Pollen (µg/kg)	Nectar (µg/kg)	Pollen (µg/kg)	Honey		
1	Sunflower	0.595	51.8	1.5/5*	Imidacloprid		ND	ND	ND		Field	Schmuck (1999)/Germany
					Monohydroxy metabolite							
					Olefin metabolite							
2	Sunflower	0.595	44.6	1.5/5*	Imidacloprid		ND	ND	ND		Field	Schmuck (1999)/Germany
					Monohydroxy metabolite							
					Olefin metabolite							
3	Sunflower	0.246	14.7	1.5/5*	Imidacloprid			ND	ND		Field	Stadler (2000)/Argentina
					Monohydroxy metabolite							
					Olefin metabolite							
4	Sunflower	0.787			Imidacloprid	1.9	3.3				Greenhouse	Stork (1999)
					Monohydroxy metabolite							
					Olefin metabolite							
5	Maize	0.89	89.2	1.5/5*	Imidacloprid		1.5<R<5/ND				Field	Schmuck (1999)/Germany
					Monohydroxy metabolite							
					Olefin							

					metabolite							
6	Maize	0.89	89.2	1.5/5*	Imidacloprid		1.5<R<5				Field	Schmuck (1999)/Germany
					Monohydroxy metabolite							
					Olefin metabolite							
7	Rape		33.5	1.5/5*	Imidacloprid	1.5<R<			1.5 <R< 5		Field	Schmuck (1999)/Germany
					Monohydroxy metabolite							
					Olefin metabolite							
8	Rape		72.1	1.5/5*	Imidacloprid	1.5<R<5			1.5 <R< 5		Field	Schmuck (1999)/Germany
					Monohydroxy metabolite							
					Olefin metabolite							
9	Rape		31.4	1.5/5*	Imidacloprid	ND	ND	1.5<R<5	ND	ND	Field	Schur (2002)/Germany
					Monohydroxy metabolite							
					Olefin metabolite							
10	Canola			0.3/1	Imidacloprid			0.3<R<1	0.3<R<1		Field	Scott-Dupree /Canada
					Monohydroxy metabolite							
					Olefin metabolite							
11	Canola		48.8	0.3/1	Imidacloprid			0.6–0.81	4.4–7.6		Field	Scott-Dupree /USA
					Monohydroxy metabolite							
					Olefin metabolite							
12	Rape			?/10*	Imidacloprid	?<R<10		?<R< 10	? <R< 10		semi-field	Schmuck (1999)/France
					Monohydroxy metabolite							
					Olefin metabolite							

13	Rape			?/10*	Imidacloprid	?<R<10		?<R< 10			semi-field	Schmuck (1999)/Sweden
					Monohydroxy metabolite							
					Olefin metabolite							
14	Rape			?/10*	Imidacloprid	?<R<10		?<R< 10	?<R<10		semi-field	Schmuck (1999)/UK
					Monohydroxy metabolite							
					Olefin metabolite							

R: Concentration of the residue

Data missing or incomplete in DAR

*It should be noted that the values of the LOD and LOQ are very high, taking into account what we know now about the level of residues in nectar and pollen, and the high toxicity of these compounds.

Maximum values found in the scientific literature (see Residue table, part A):

Pollen loads: 5.7 µg/kg (Chauzat et al., 2011)
Pollen from the flower: 18µg/kg (Bonmatin et al., 2005)
Honey: 2 µg/kg (Chauzat et al., 2009)
Honey bees: 11.1 kg/kg (Chauzat et al., 2011)

Table G5: Residue table: data from the DAR other compounds from spray treatment

N°	Compound	Crop	Dose (g/ha)	LOD/LOQ (µg/kg)	Days after treatment	From comb/foragers		Conditions	Country Year	Reference
						Pollen (µg/kg)	Nectar (µg/kg)			
1	Teflubenzuron	Winter rape	78.75		1 4–14 7	1710 110–160	70 <10	Field	? 1987	Eichler (1987) cited in UK (2007)
			157.5		0 7	23,600 150		Tent	? 1987	
2	Flufenoxuron	*Phacelia*	40			60–600[a] 40–730[b]	<LOQ	Tunnel	Germany 2002	Schur (2003f) cited in France (2010)
3	Flufenoxuron	*Phacelia*	40			10–320[c] 30–60[b]	LOD<R[a]< LOQ	Field	Spain 2002	Veit and Weber (2003) cited in France (2010)
4	Flufenoxuron	*Phacelia*	40			<LOQ[c]	LOD<R[a]<LOQ	Field	Italy 2002	Veit and Weber (2003) cited in France (2010)
5	Flufenoxuron	*Phacelia*	40			690[b], 3620[b] 20[c], 120[c]	LOD<R[a]< LOQ <10[b], 80[b]	Field	France 2002	Veit and Weber (2003) cited in France (2010)
6	Flufenoxuron	Grape	40			<10[b], 60[b] LOD<R[c] <LOQ	LOD<R[c]<LOQ	Field	France 2002	Veit and Weber (2003)

R: Concentration of the residue
a: "foragers – comb"
b: "foragers"
c: "comb"

										cited in France (2010)
7	Carbofuran	Maize	12 000			1–2.9				Diehl (2005) cited in Belgium (2009)

Table G6: Residue table: data from Germany (Deutsches Bienenmonitoring; von der Ohe, 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

Table G7: Residues in nectar from spray applications

Reference	Spray applications a.s.	Application rate (g a.s./ha)	Crop	LOQ (mg/kg)	Max residues (µg/kg)	Extrapolation (ng/g)	Application of 1 kg/ha (mg/kg)
Schatz and Wallner (2009)	Azoxystrobin	250	WOSR	0.001	1 450	5 800	5.8
Schatz and Wallner (2009)	Azoxystrobin	250	WOSR	0.001	1 000	4 000	4.0
Schatz and Wallner (2009)	Boscalid	200	WOSR	0.001	200	1 000	1.0
Schatz and Wallner (2009)	Boscalid	200	WOSR	0.001	200	1 000	1.0
Schatz and Wallner (2009)	Boscalid	250	WOSR	0.001	1 600	6 400	6.4
Schatz and Wallner (2009)	Boscalid	250	WOSR	0.001	1200	4 800	4.8
Schatz and Wallner (2009)	Dimoxystrobin	200	WOSR	0.001	330	1 650	1.7
Schatz and Wallner (2009)	Dimoxystrobin	200	WOSR	0.001	330	1 650	1.7
Schatz and Wallner (2009)	Iprodion	255	WOSR	0.05	1 450	5 686	5.7
Schatz and Wallner (2009)	Metconazole	90	WOSR	0.001	330	3 667	3.7
Schatz and Wallner (2009)	Thiophanat-methyl	500	WOSR	0.005	500	1 000	1.0
Schatz and Wallner (2009)	Thiophanat-methyl	500	WOSR	0.005	400	800	0.8
Schatz and Wallner (2009)	Carbendazim met.	500	WOSR	0.001	650	1 300	1.3
Schatz and Wallner (2009)	Carbendazim met.	500	WOSR	0.001	650	1 300	1.3
Schatz and Wallner (2009)	Sum TP+C	500	WOSR	–	1 150	2 300	2.3
Schatz and Wallner (2009)	Sum TP+C	500	WOSR	–	1 050	2 100	2.1
Schatz and Wallner (2009)	Fluvalinat	32	WOSR	0.01	170	5 313	5.3
Schatz and Wallner (2009)	Fluvalinat	32	WOSR	0.01	400	12 500	12.5
Schatz and Wallner (2009)	Thiacloprid	72	WOSR	0.001	36	500	0.5

Schatz and Wallner (2009)	Thiacloprid	72	WOSR	0.001	9	125	0.1
Schatz and Wallner (2009)	Prothioconazole	175	WOSR	0.01	25	143	0.1
Data from DAR	Teflubenzuron	78.75	Phacelia	Not given	70	889	0.9
Data from DAR	Flufenoxuron	40	Grape	Not given	80	2 000	2.0
Choudhary and Sharma (2008)	Endosulfan	525	Mustard	Not given	1 825	3 476	3.5
Choudhary and Sharma (2008)	Lambda-cyhalothrin	75	Mustard	Not given	909	12 120	12.1
Choudhary and Sharma (2008)	Spiromesifen	225	Mustard	Not given	1 540	6 844	6.8
Wallner (2009)	Boscalid	500	WOSR	0.001	1430	2860	2.9
Wallner (2009)	Prothioconazole	250	WOSR	0.001	690	2760	2.8
Data from DAR	Chloranthraniliprole	60	Phacelia	0.001	33	550	0.6
Anonymous[1], 2011	*Pyrethroid	4.8	WOSR	>0.01	11	2 292	2.3

[1]Name of the author cannot be given due to confidentiality.
*Substance class.
WOSR, winter oilseed rape.

Table G8: Residues in nectar from systemic seed treatments

References (see tables G2–G4)/Trial No	Seed treatment a.s.	Application rate (g a.s./ha)	Crop	LOQ (mg/kg)	Max residues (µg/kg)	Extrapolation (ng/g)	Application of 1 kg/ha (mg/kg)
1	Clothianidin	27	SOSR	0.001	3	111	0.11
1	Clothianidin metabolite TZMU	27	SOSR	0.001	1	37	0.04
1	Clothianidin metabolite TZNG	27	SOSR	0.001	0	0	0.00
2	Clothianidin	27	SOSR	0.001	5.4	200	0.20
2	Clothianidin metabolite TZMU	27	SOSR	0.001	1	37	0.04
2	Clothianidin metabolite TZNG	27	SOSR	0.001	1	0	0.00
7	Clothianidin	49.8	WOSR	0.001	1	20	0.02
7	Clothianidin metabolite TZMU	49.8	WOSR	n.a.	0	0	0.00
7	Clothianidin-metabolite TZNG	49.8	WOSR	n.a.	0	0	0.00
9	Clothianidin	49.8	SOSR	n.a.	8.6	173	0.17
9	Clothianidin	49.8	SOSR	n.a.	7.2	145	0.14
11	Clothianidin	49.8	SOSR	n.a.	1	20	0.02
11	Clothianidin	49.8	SOSR	n.a.	1	20	0.02
12a	Clothianidin	42	WOSR	n.a.	1.1	26	0.03

SOSR, summer oilseed rape; WOSR, winter oilseed rape. n.a., not available (data missing or incomplete in the DAR).

Table G9: Residues in honey from spray applications

Sources	Spray applications (a.s.)	Application rate (g a.s./ha)	Crop	LOQ (mg/kg)	Max residues (µg/kg)	Extrapolation (ng/g)	Application of 1 kg/ha (mg/kg)
Schatz and Wallner (2009)	Boscalid	200	WOSR	0.001	63.1	316	0.32
Schatz and Wallner (2009)	Dimoxystrobin	200	WOSR	0.001	5.6	28	0.03

Schatz and Wallner (2009)	Prothiconazole	175	WOSR	0.01	10	57	0.06
Schatz and Wallner (2009)	Thiophanate-methyl	500	WOSR	0.005	43.8	88	0.09
Schatz and Wallner (2009)	Metabolite carbendazim	500	WOSR	0.001	405	810	0.81
Schatz and Wallner (2009)	Azoxystrobin	250	WOSR	0.001	174	696	0.70
Schatz and Wallner (2009)	Tau-fluvalinate	32	WOSR	0.01	17.5	547	0.55
Schatz and Wallner (2009)	Thiacloprid	72	WOSR	0.001	1.8	25	0.03

Table G10: Residues in pollen from spray applications

Reference/trial no (see Table G5)	Spray applications (a.s.)	Crop	Application rate (g a.s./ha)	LOQ (mg/kg)		Extrapolation (ng/g)	Application of 1 kg/ha (mg/kg)
Choudhary and Sharma (2008)	Endosulfan	Mustard	525	n.a.	2224	4236.2	4.2
Choudhary and Sharma (2008)	Endosulfan	Mustard	525	n.a.	2127	4051.4	4.1
Choudhary and Sharma (2008)	Lambda-Cyhalothrin	Mustard	75	n.a.	1672	22293.3	22.3
Choudhary and Sharma (2008)	Lambda-cyhalothrin	Mustard	75	n.a.	1612	21493.3	21.5
Choudhary and Sharma (2008)	Spiromesifen	Mustard	225	n.a.	2101	9337.8	9.3
Choudhary and Sharma (2008)	Spiromesifen	Mustard	225	n.a.	1827	8120.0	8.1
Wallner (2009)	Boscalid	WOSR	500	0.001	26200	52 400.0	52.4
Wallner (2009)	Prothioconazole	WOSR	250	0.001	1	4.0	0.004
Data from DAR	Chloranthraniliprole	WOSR	60	0.001	2600	43 333.3	43.3
Data from DAR/1	Teflubenzuron	*Phacelia*	78.75	n.a.	1710	21 714.3	21.7
Data from DAR/1	Teflubenzuron	*Phacelia*	157.5	n.a.	23600	14 9841.3	149.8
Data from DAR/6	Flufenoxuron	Grape	40	n.a.	730	18 250.0	18.3
Data from DAR/6	Flufenoxuron	Grape	40	n.a.	3620	90 500.0	90.5
Data from DAR/7	Carbofuran	Maize	12 000	n.a.	2.9	0.2	0.0002
Anonymous[1]	*Pyrethroid	WOSR	4.8	0.01	102.3	21 312.5	21.3
Kubik et al. (2000)	Difenconazole	Apple	200	0.01	411.0	2 055.0	2.1
Kubik et al. (2000)	Captan	Apple	2 000	0.01	80 90.0	4 045.0	4.0
Kubik et al. (1999)	Vinclozolin	Cherry	375	0.004	27 218.0	72 581.3	72.6

[1]Name of the author cannot be given owing to uncertainties concerning confidentiality.
*Substance class.
WOSR, winter oilseed rape; n.a., not available (data missing or incomplete in the DAR).

Table G11: Residues in pollen collected by bees, collected from flowers or bee bread (see tables G2-G4 for details), systemic seed treatments

References (see tables G2–G4)/trial no	Seed treatments (a.s.)	Crop	Application rate (g a.s./ha)	LOQ (mg/kg)	Max residues (µg/kg)	Extrapolation (ng/g)	Application of 1 kg/ha (mg/kg)
11	Imidacloprid + metabolites	SOSR	48.8	0.001	7.6	156	0.16
5	Imidacloprid	Maize	89.2	0,005	5*	56.05	0.06

6	Imidacloprid	Maize	89.2	0,005	5*	56.05	0.06
7	Imidacloprid	Rape	33.5	0,005	5*	149.25	0.15
8	Imidacloprid	Rape	72.1	0,005	5*	69.35	0.04
E	Thiamethoxam	Sunflower	26	0.001	1*	38.46	0.04
E	CGA322704	Sunflower	26	n.a.	1*	38.46	0.04
F	Thiamethoxam	WOSR	26	0.001	4.2	161.54	0.16
F	CGA322704	WOSR	26	n.a.	1*	38.46	0.04
G	Thiamethoxam	WOSR	29	0.001	4.6	158.62	0.16
G	CGA322704	WOSR	29	n.a.	1*	34.48	0.03
H	Thiamethoxam	Sunflower	28	0.001	1.1	39.29	0.04
H	CGA322704	Sunflower	28	n.a.	1*	35.71	0.04
I	Thiamethoxam	Sunflower	22	0.001	3.2	145.45	0.15
I	CGA322704	Sunflower	22	n.a.	1*	45.45	0.05
J	Thiamethoxam	Sunflower	19	0.001	1*	52.63	0.05
J	CGA322704	Sunflower	19	n.a.	1*	52.63	0.05
K	Thiamethoxam	Sunflower	18	0.001	1*	55.56	0.06
K	CGA322704	Sunflower	18	n.a.	1*	55.56	0.06
M	Thiamethoxam	WOSR	31	0.001	3.6	116.13	0.12
M	CGA322704	WOSR	31	n.a.	1*	32.26	0.03
O	Thiamethoxam	WOSR	27	n.a.	0.7	25.93	0.03
O	CGA322704	WOSR	27	n.a.	0.24	8.89	0.01
2	Clothianidin	SOSR	27	0.001	2.5	93	0.09
2	TZMU	SOSR	27	0.001	0	0	0.00
2	TZNG	SOSR	27	0.001	0	0	0.00
7	Clothianidin	WOSR	49.8	0.001	1c	20	0.02
7	TZMU	WOSR	49.8	n.a.	0	0	0.00
7	TZNG	WOSR	49.8	n.a	0	0	0.00
9	Clothianidin	SOSR	49.8	n.a.	4.1	82	0.08
10	Clothianidin	SOSR	49.8	n.a.	3.3	66	0.07
11	Clothianidin	SOSR	49.8	n.a.	1	20	0.02
12a	Clothianidin	WOSR	42	n.a.	3	71	0.07
12b	Clothianidin	WOSR	30	n.a.	2.8	93	0.09
3	Clothianidin	Sunflower	25.4	0.001	3.1	122	0.12
3	TZMU	Sunflower	25.4	n.a.	1*	39	0.04
3	TZNG	Sunflower	25.4	n.a.	1*	39	0.04
4	Clothianidin	Sunflower	25.4	n.a.	2.9	114	0.11
4	TZMU	Sunflower	25.4	n.a.	1*	39	0.04
4	TZNG	Sunflower	25.4	n.a.	1*	39	0.04
Nikolakis et al. (2009)	Clothianidin	Maize	125		10.4	83	0.08
8	Clothianidin	Maize	53.8	n.a.	6.2	115	0.12
8	TZMU	Maize	53.8	n.a.		0	0.00
8	TZNG	Maize	53.8	n.a.	1*	19	0.02

8b	Clothianidin	Maize	53.8	n.a.	2.9	54	0.05

*If the residue detected was between LOD (e.g. 0.3) and LOQ (e.g. 1) , we made the worst case assumption that the residue was equal to the LOQ (1 in this case) for calculation
n.a., not available (data missing or incomplete in the DAR).

H. RESIDUES IN GUTTATION DROPLETS

We summarised the data in the literature on the concentration of residues in guttation droplets.

In all experiments in the APENET project (Girolami et al., 2009; CRA-API 2009, 2010), the guttated water of plants germinated from seeds dressed with pesticides contains the active ingredients with wide concentration range: from 0.1 to >300 mg/L depending on the crop, the substance, the environmental conditions, the days from plant emergence and the time of day on which sampling took place (Table H1).

Table H1: Residue of insecticides found in maize guttation drops in APENET project

Seed	Active ingredient	Sampling period (days after sowing or emergence/ phenological phase)	Sampling time	Water availability in the soil/soil characteristics	Environmental conditions	Residue concentration (mg/L)	Reference
Gaucho (0.5 mg/seed)	Imidacloprid	In the first 3 weeks from emergence	Not specified	Not specified	In laboratory	47 ± 9.96	Girolami et al. (2009), CRA-API (2009)
Poncho (1.25 mg/seed)	Clothianidin	In the first 3 weeks from emergence	Not specified	Not specified	In laboratory	23.3 ± 4.2	Girolami et al. (2009), CRA-API (2009)
Cruiser (1 mg/seed)	Thiametoxam	In the first 3 weeks from emergence	Not specified	Not specified	In laboratory	11.9 ± 3.32	Girolami et al. (2009), CRA-API (2009)
Poncho (1.25 mg/seed)	Clothianidin	For 2 weeks, after 1 month from sowing	6.00–8.00 AM	Not specified	In field	0.28 ± 0.10	Sgolastra et al. (2010b), CRA-API (2009)
Gaucho (0.5 mg/seed)	Imidacloprid	Seedling	Not specified	Not specified	In laboratory: 27 ± 1 °C; 90–95 % RH; L:D = 16:8	49.9	Unpublished, APENET project
Gaucho (0.5 mg/seed)	Imidacloprid	Young plant with 1–2 leaves	Not specified	Not specified	In laboratory: 27±1 °C; 90–95 % RH; L:D = 16:8	26	Unpublished, APENET project
Poncho (1.25 mg/seed)	Clothianidin	Seedling	Not specified	Not specified	In laboratory: 27±1 °C; 90–95 % RH; L:D = 16:8	35.6	Unpublished, APENET project
Poncho (1.25 mg/seed)	Clothianidin	Young plant with 1–2 leaves	Not specified	Not specified	In laboratory: 27 ± 1 °C; 90–95 % RH; L:D = 16:8	19.8	Unpublished, APENET project
Cruiser (1 mg/seed)	Thiametoxam	Seedling	Not specified	Not specified	In laboratory: 27 ± 1 °C; 90–95 % RH; L:D = 16:8	47.05	Unpublished, APENET project
Cruiser (1 mg/seed)	Thiametoxam	Young plant with 1–2 leaves	Not specified	Not specified	In laboratory: 27 ± 1 °C; 90–95 % RH; L:D = 16:8	26.9	Unpublished, APENET project

Regent (1 mg/seed)	Fipronil	Seedling	Not specified	Not specified	In laboratory: 27 ± 1 °C; 90–95 % RH; L:D = 16:8	77	Unpublished, APENET project
Regent (1 mg/seed)	Fipronil	Young plant with 1–2 leaves	Not specified	Not specified	In laboratory: 27 ± 1 °C; 90–95 % RH; L:D = 16:8	46.27	Unpublished, APENET project
Gaucho (0.5 mg/seed)	Imidacloprid	1 day from emergence	8.00–11.00 AM	Not specified	In field	128.4	CRA-API (2010)
Gaucho (0.5 mg/seed)	Imidacloprid	8 days from emergence	8.00–11.00 AM	Not specified	In field	1.0	CRA-API (2010)
Gaucho (0.5 mg/seed)	Imidacloprid	15 days from emergence	8.00–11.00 AM	Not specified	In field	0.8	CRA-API (2010)
Gaucho (0.5 mg/seed)	Imidacloprid	18 days from emergence	8.00–11.00 AM	Not specified	In field	0.5	CRA-API (2010)
Gaucho (0.5 mg/seed)	Imidacloprid	19 days from emergence	8.00–11.00 AM	Not specified	In field	0.4	CRA-API (2010)
Gaucho (0.5 mg/seed)	Imidacloprid	22 days from emergence	8.00–11.00 AM	Not specified	In field	0.2	CRA-API (2010)
Poncho (1.25 mg/seed)	Clothianidin	1 day from emergence	8.00–11.00 AM	Not specified	In field	31.9	CRA-API (2010)
Poncho (1.25 mg/seed)	Clothianidin	8 days from emergence	8.00–11.00 AM	Not specified	In field	1.0	CRA-API (2010)
Poncho (1.25 mg/seed)	Clothianidin	15 days from emergence	8.00–11.00 AM	Not specified	In field	1.0	CRA-API (2010)
Poncho (1.25 mg/seed)	Clothianidin	18 days from emergence	8.00–11.00 AM	Not specified	In field	1.1	CRA-API (2010)
Poncho (1.25 mg/seed)	Clothianidin	19 days from emergence	8.00–11.00 AM	Not specified	In field	0.8	CRA-API (2010)
Poncho (1.25 mg/seed)	Clothianidin	22 days from emergence	8.00–11.00 AM	Not specified	In field	0.6	CRA-API (2010)
Cruiser (1 mg/seed)	Thiametoxam	1 day from emergence	8.00–11.00 AM	Not specified	In field	172.3	CRA-API (2010)
Cruiser (1 mg/seed)	Thiametoxam	8 days from emergence	8.00–11.00 AM	Not specified	In field	2.4	CRA-API (2010)
Cruiser (1 mg/seed)	Thiametoxam	15 days from emergence	8.00–11.00 AM	Not specified	In field	0.4	CRA-API (2010)
Cruiser (1 mg/seed)	Thiametoxam	18 days from emergence	8.00–11.00 AM	Not specified	In field	0.2	CRA-API (2010)
Cruiser (1 mg/seed)	Thiametoxam	19 days from emergence	8.00–11.00 AM	Not specified	In field	0.3	CRA-API (2010)
Cruiser (1 mg/seed)	Thiametoxam	22 days from emergence	8.00–11.00 AM	Not specified	In field	0.1	CRA-API (2010)
Poncho (1.25 mg/seed)	Clothianidin	15 days from emergence	8.00–11.00 AM	Not specified	In field	1.02	CRA-API (2010)
Poncho (1.25 mg/seed)	Clothianidin	15 days from emergence	8.00–11.00 AM	Not specified	In field but plant protected with a sheet of Plexiglas	2.38	CRA-API (2010)
Gaucho (0.5 mg/seed)	Imidacloprid	15 days from emergence	8.00–11.00 AM	Not specified	In field	0.83	CRA-API (2010)
Gaucho (0.5 mg/seed)	Imidacloprid	15 days from emergence	8.00–11.00 AM	Not specified	In field but plant protected with a sheet of Plexiglas	1.17	CRA-API (2010)
Gaucho (0.5 mg/seed)	Imidacloprid	From 1 to 20 days from emergence	Not indicated	Dry (50 l/22.5 m^2)	Screening tunnel	124.38±44.35	CRA-API (2010)
Gaucho (0.5 mg/seed)	Imidacloprid	From 1 to 20 days from emergence	Not indicated	Damp (1 100 L/22.5 m^2)	Screening tunnel	204.54 ± 91.76	CRA-API (2010)
Gaucho (0.5 mg/seed)	Imidacloprid	From 1 to 20 days from emergence	Not indicated	Wet (2 500 L/22.5 m^2)	Screening tunnel	231.92 ± 80.64	CRA-API (2010)
Poncho (1.25 mg/seed)	Clothianidin	From 1 to 20 days from emergence	Not indicated	Dry (50 L/22.5 m^2)	Screening tunnel	64.91 ± 25.68	CRA-API (2010)
Poncho (1.25 mg/seed)	Clothianidin	From 1 to 20 days from emergence	Not indicated	Damp (1 100 l/22.5 m^2)	Screening tunnel	82.11 ± 33.01	CRA-API (2010)
Poncho (1.25 mg/seed)	Clothianidin	From 1 to 20 days from emergence	Not indicated	Wet (25 00 L/22.5 m^2)	Screening tunnel	90.60 ± 27.27	CRA-API (2010)

Cruiser (1 mg/seed)	Thiametoxam	From 1 to 20 days from emergence	Not indicated	Dry (50 l/22.5 m^2)	Screening tunnel	342.10 ± 178.58	CRA-API (2010)
Cruiser (1 mg/seed)	Thiametoxam	From 1 to 20 days from emergence	Not indicated	Damp (1 100 L/22.5 m^2)	Screening tunnel	115.24 ± 26.00	CRA-API (2010)
Cruiser (1 mg/seed)	Thiametoxam	From 1 to 20 days from emergence	Not indicated	Wet (2 500 L/22.5 m^2)	Screening tunnel	71.88 ± 17.80	CRA-API (2010)

The excretion of guttation droplets occurs regularly in monocotyledons, is periodical in dicotyledons (e.g. in rape but only rarely in sugar beet) and usually occurs at high humidity conditions.

The higher values of residues concentrations were observed in the days after the crop emergence. Later, this concentration decreased but remained detectable over several weeks (see Figures H1–6 and Reetz et al., 2011).

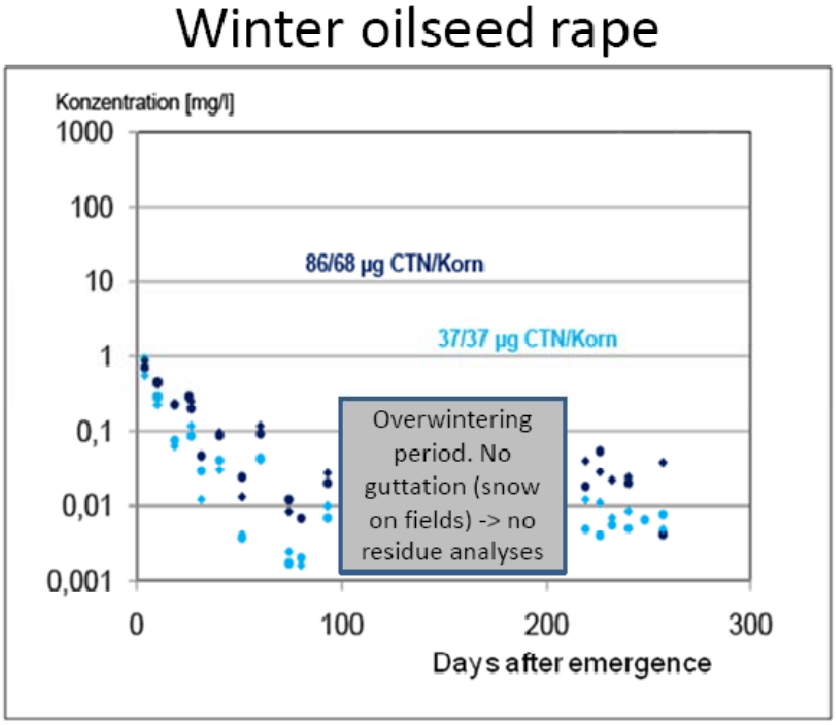


Figure H1: Pesticide concentrations in guttation droplets collected from winter oilseed rape before and after overwintering (Schenke et al., 2010).

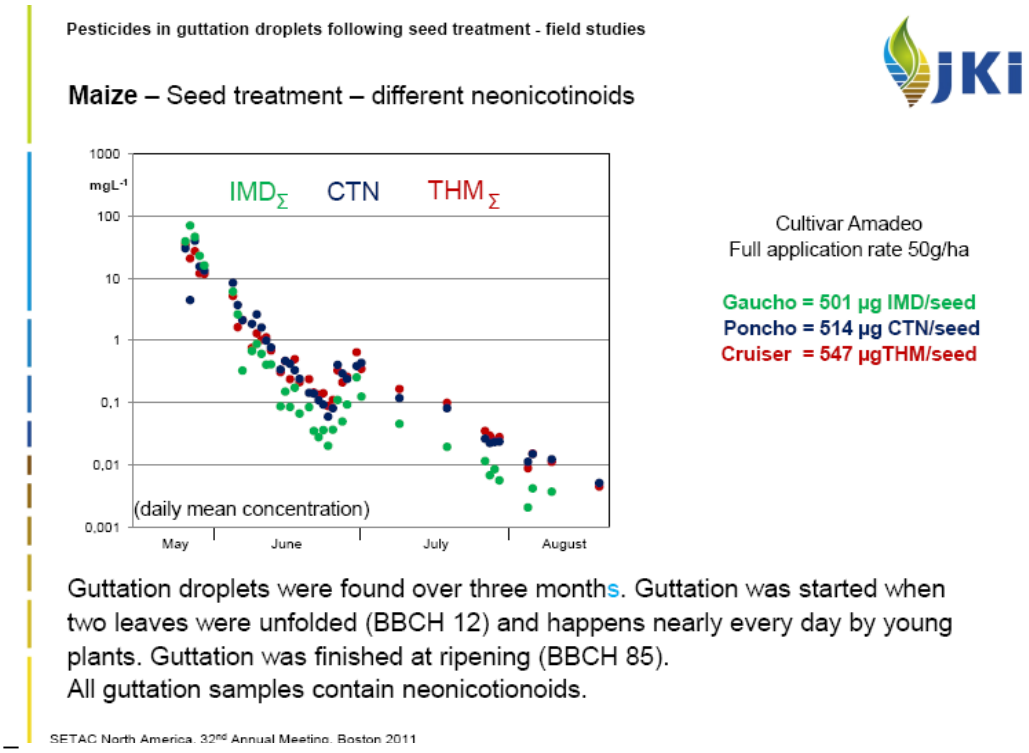


Figure H2: Concentrations of clothianidin, imidacloprid and thiamethoxam in guttation droplets following different seed treatments of maize collected over several weeks under field conditions (Schenke et al., 2011).

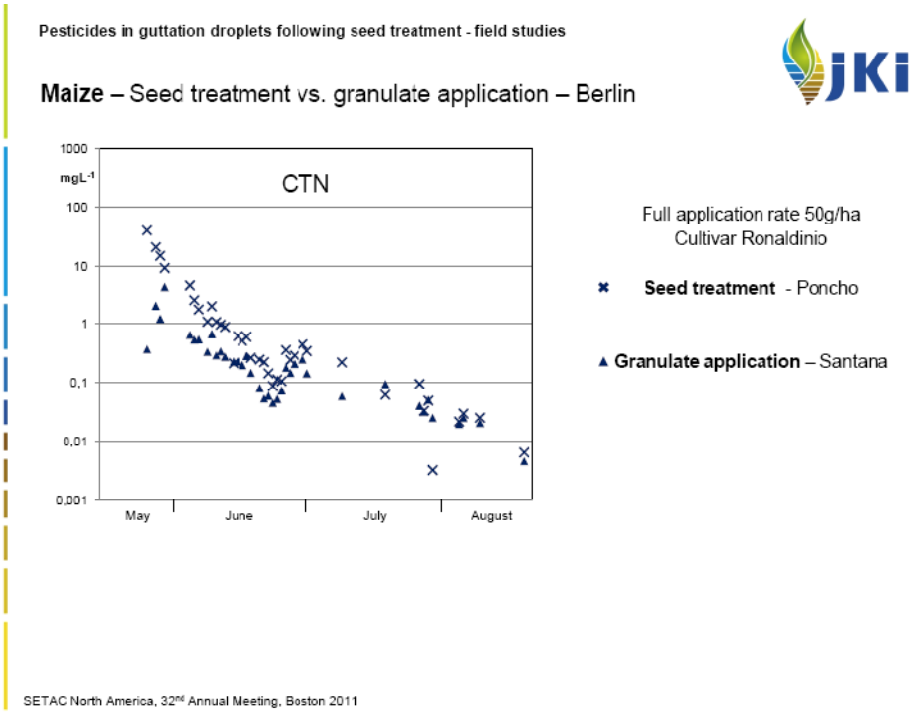


Figure H3: Concentrations of clothianidin in guttation droplets following seed treatment of maize or a granular application collected over several weeks under field conditions (Schenke et al., 2011).

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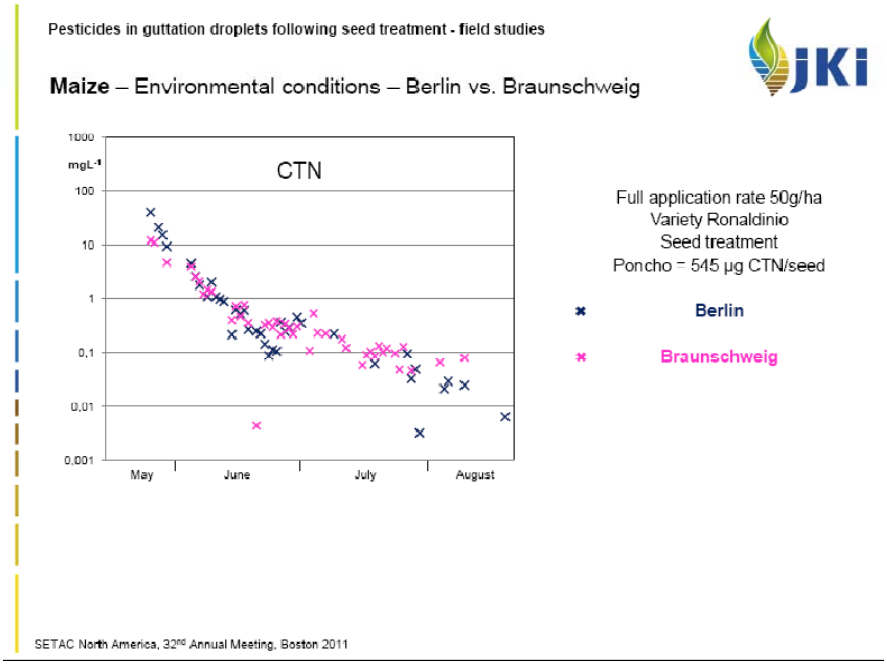

Figure H4: Pesticide concentrations in guttation under field conditions collected in two different environmental conditions (Schenke et al., 2011).

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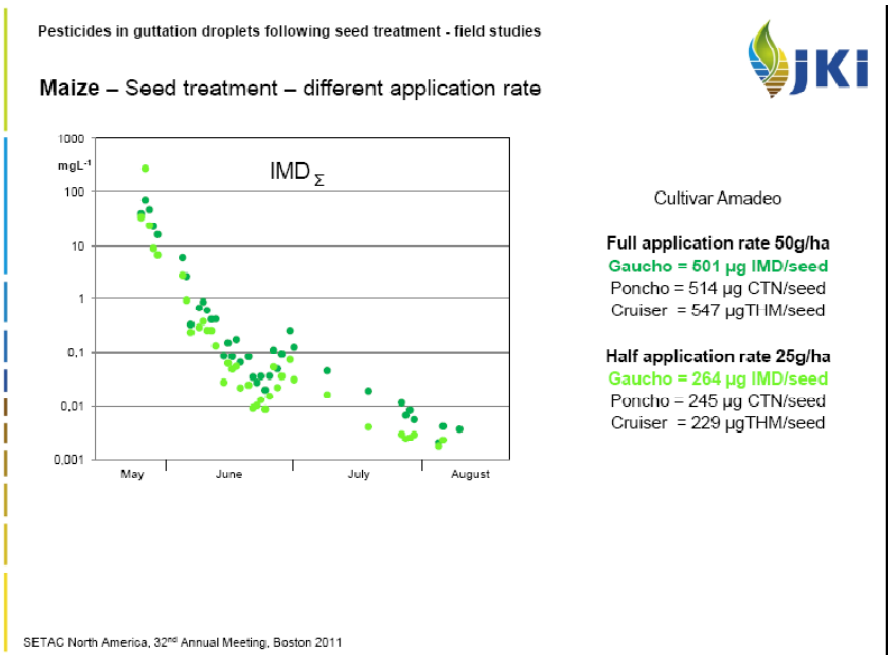

Figure H5: Pesticide concentrations in guttation under field conditions collected in two different environmental conditions (Schenke et al., 2011).

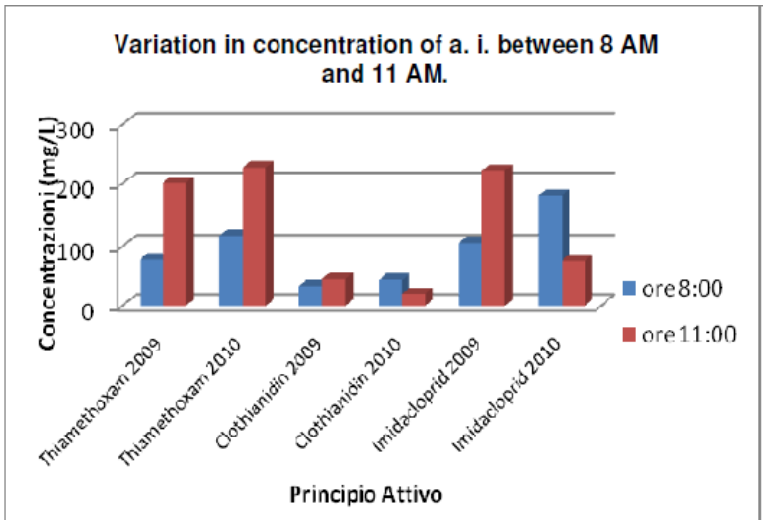

Figure H6: Pesticide concentrations in guttation droplets from maize collected at two different times on the same day (CRA-API, 2010).

Analyses of guttation samples collected at different times on the specified day, and analysed at the Department of Chemistry of the University of Padova, showed variations in the concentration of the active ingredient (Figure H7).

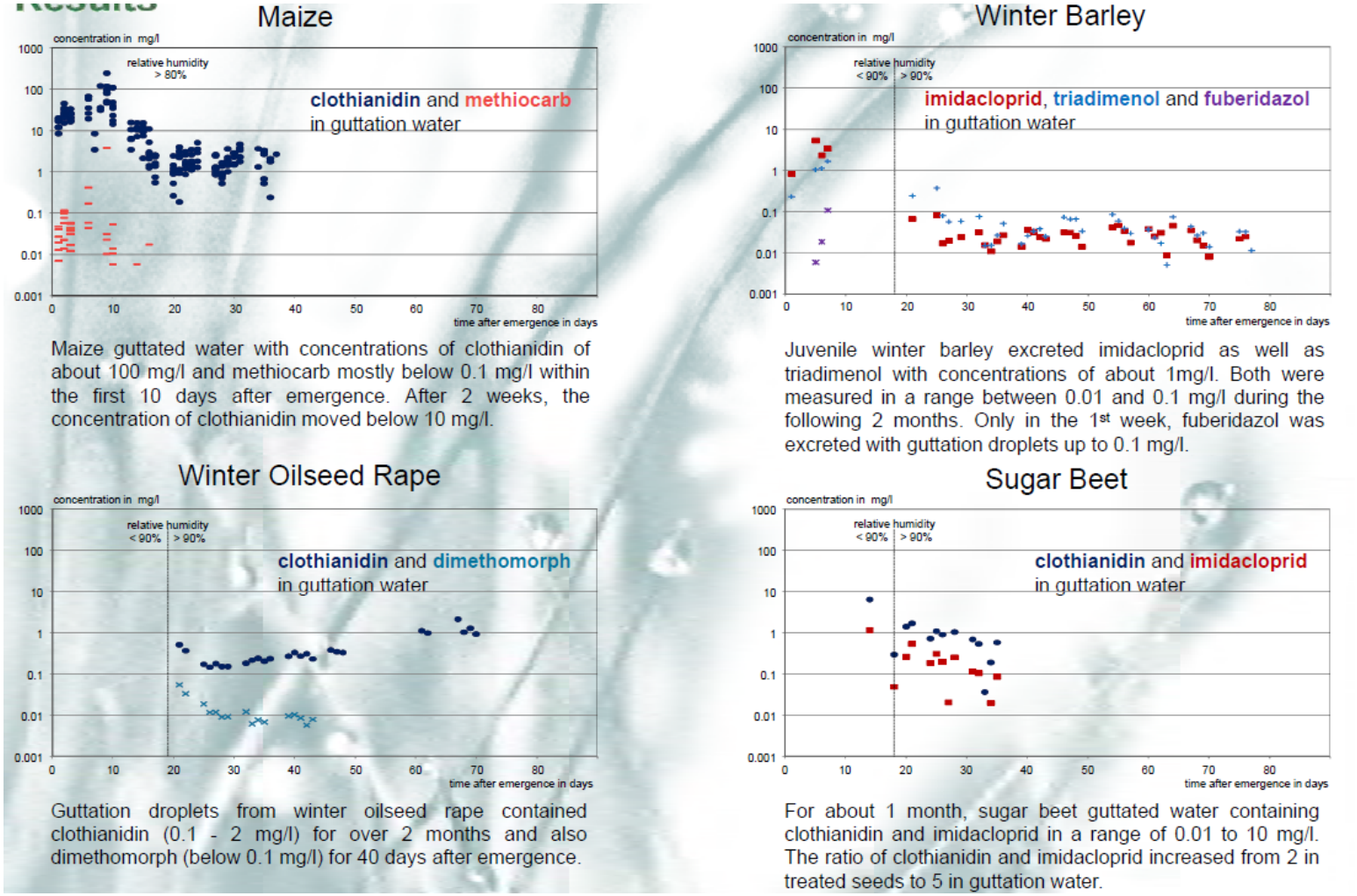

Figure H7: Pesticide concentrations in guttation droplets collected from maize, winter barley, winter oilseed rape and sugar beet over several weeks under greenhouse conditions (Schenke et al., 2010).

The persistence of the different active ingredients used in the maize seed coating process showed that imidacloprid and thiamethoxam dispersed through the plants via the xylem flow to a greater extent than clothianidin. This finding is basically in line with the water solubility of these active ingredients (clothianidin is more hydrophobic), given that the xylem is a fundamentally watery medium (CRA-API, 2010).

A greater presence of active ingredients was noted in the plants that were protected against the rain (i.e. when the plant was protected with a sheet of Plexiglas). This was probably not only because the active ingredient had been subjected to a lesser degree to leaching, but also because the different microclimate under the Plexiglas resulted in guttation and therefore a higher concentration in the guttation droplets (Table H1).

Residue concentration in maize guttation droplets was influenced by different soil irrigation regimes. In a dry regimen, the appearance of guttation is delayed. With greater rainfall, a diluting effect is observed, so that the concentration in soil water is reduced. This effect is more marked for thiamethoxam, which is the most water-soluble compound among those considered. Accordingly, thiamethoxam was found to be the most concentrated active ingredient in the plants grown under a dry water regime. Thus, in a damp/wet regime, the active ingredient concentration appears to be in line with the polarity of the compound (Table H1).

Risk for bees in realistic conditions

In general, the risk for bee colonies is likely to decrease rapidly with distance of the colonies to treated crops showing guttation. The risk may also be strongly influenced by the availability or absence of alternative water sources nearby. Whereas nectar and pollen are highly attractive for bees and will be foraged in a coordinated way and attract bees from further away, guttation droplets are only one of several possible water sources in the surrounding of a colony and usually available for only a limited time period in the morning and not every day.

Plants offering nectar and pollen will attract bees from further away, whereas water is collected in closer proximity of the hive. Thus, in contrast to nectar and pollen, collection of guttation liquid does not appear to be a regular exposure scenario.

The possible uptake of guttation water may be highly variable and is determined by, for example, climate conditions, time of bee activity, seasonal activity and the seasonal water needs of colonies and the occurrence of guttation droplets containing high residue levels. The water need of a colony is highest during spring and summer.

As water foragers will preferably choose water sources in the proximity of the hive and avoid long-distance flights for energetic reasons, the position of the bee hive in relation to the treated crop and the availability of alternative water sources are most important factors. Furthermore, if guttation occurs, it also occurs in untreated plants like grasses and weeds.

Potential information from laboratory studies, field studies, monitorings

In laboratory studies it is not possible to stimulate the uptake of guttation liquid or pure water without adding sugar. Guttation liquid artificially spiked with sucrose is used as a carbohydrate source. At the moment, laboratory feeding of bees represents an unrealistic and artificial exposure scenario. Thereforeit is inadequate to assess a risk for bees but suitable for fast screening of guttation during feeding tests in cages. The outcomes of such tests have shown to be comparable to OECD 213/214 laboratory toxicity data, resulting in high mortality after feeding sugar-enriched guttation droplets of maize.

In semi-field studies, controlled conditions in tents offer the possibility to simulate water collection from guttation droplets and other water sources. Alternative water sources can be excluded to ensure

maximum exposure. The effects on foragers and hive bees and different brood stages can be measured in worst-case exposure scenarios. Nevertheless, semi-field studies have a limited potential for extrapolation of the findings to field conditions.

In field studies bees can freely choose water sources. Field studies can be designed to cover different scenarios from realistic field conditions to artificially aggravated exposure. In field trials, it is difficult to draw conclusions on the activity of water-foraging bees in the surrounding environment and to estimate the portion of water foragers using guttation droplets or other sources, and there is no control over the intensity of use of focused water sources. Also the assessments are very labour intensive. Behaviour of foragers, effects on foragers, hive bees and different brood stages, brood development and colony development can be assessed under realistic worst-case exposure conditions.

Monitoring studies offer a wide range of possible designs to estimate the effects on bee colonies. The significance of the results depends on the design of the study. As the colonies show individual water-foraging behaviour and the environmental conditions of the study sites may be variable, the intrinsic variability of the systems can be compensated by appropriate replicate (e.g. colony and field) numbers.

Available information from laboratory studies, field studies, monitorings

Some monitoring studies have been conducted by industry and research institutes. For granular application with the a.s. Clothianidin bee monitorings were conducted in 2010 and 2011 in different regions of Germany by the apicultural state institute LWG Veitshöchsheim (2010 and 2011) and the bee institute LAVES Celle (2010 and 2011) and the DLR (2011). Colonies were set up at the field border before emergence of the maize crops. At the location in Veitshöchsheim, in both 2010 and 2011 (Illies et al., 2011), and also in Rhineland Palatinate (Schulz, 2011), no remarkable mortality peaks were seen and it was concluded that mortality and brood and colony development were at a normal level during the whole study and no treatment-related effects were seen. In samples from days with no increased mortality, residues were also found, indicating that single bees came in contact with the active substance but not leading to an overall increase of mortality (Illies et al., 2011).

Also, in the monitoring by LAVES, no remarkable mortality peaks were seen in 2011 and it was concluded that mortality and brood and colony development were at a normal level and no treatment-related effects were observed during the whole study in 2011 (von der Ohe, unpublished); nevertheless, in the trial 2010, events of clearly increased mortality were observed and residues of clothianidin were found in the dead bees (von der Ohe, 2010). It was concluded that the mortality was caused by uptake of guttation fluid. Although guttation occurred frequently during this trial, use of guttation fluids leading to increased mortality did not occur regularly but only on single events. As no mortality peaks were seen in the other maize monitoring trials, although guttation frequently occurred, it can be concluded that the use of larger amounts of guttation fluids by a larger number of bees occurs only in special circumstances. The high variability of effects observed under practical conditions is due to the individual location, climate conditions, water availability and water need.

During the guttation period in maize and wheat fields, no bees were observed collecting guttation drops (Sgolastra et al., 2010b; Reetz et al., 2011). Also, in a monitoring trial with seed treated maize in 2011 and also in winter oilseed rape 2010 and 2011 by the Julius Kühn-Institute-JKI (Pistorius, unpublished) no treatment-related mortality peaks were observed.

Likewise, in incident reporting schemes of different countries no apparent poisoning incidents linked with guttation were reported by beekeepers or ascertained in the national incident investigation schemes. Nevertheless, crops with potential high risk, e.g. crops like maize, frequently showing guttation with highest peak residues up to 100 mg/L at time of high water need of colonies (e.g. seed treatments with neonicotinoids) were not authorised in these countries, so from incident data no conclusion can be drawn. For other crops in some countries, e.g. Germany and the UK, winter oilseed rape or sugar beet crops seed treated with neonicotinoids have been registered for more than 10 years and no link with bee poisoning incidents was concluded in the national investigation schemes

(Pistorius and Thompson, personal communication). At emergence of winter oilseed rape, brood activity and colony strength are declining and bee activity is generally lower and peak residues of neonicotinoids in guttation droplets reached approximately 1 mg/L at time of emergence for winter oilseed rape.

Regarding the risk for bee colonies, so far it is possible to draw some conclusions for different seed treatments and different crops on the basis of frequency and occurrence, the expected residues and the time of occurrence of residues in the season. For example for sugar beets it was concluded the potential risk is rather low, as guttation occurs less frequent and droplets are small although the guttation period with peak residues is in spring/summer. For winter oilseed rape residues are highest in autumn and clearly lower in the spring, the time when bees have a high water need. Hop seems not to produce guttation droplets at all (Engelhardt et al., 2011). It seems that cereals, and especially maize, which a higher seed loading of active ingredient per seed seem to be crops with highest potential risk.

It is assumed the water need of honey bees is likely to be higher than for bumble bees; as complex regulation of the honey bee brood nest humidity is performed, the larval food has high water content and stored honey may need to be diluted with water for the preparation.

Data from experiments with intrinsically highly toxic, systemic insecticides indicate that further studies exceeding standard laboratory toxicity data might only be needed for a limited number of actives. Criteria for active ingredients that may trigger consideration are systemic properties of active substance (xylem mobility), persistence, intrinsic toxicity for bees, mode of action. Regulatory decisions should be done on a case by case basis and exposure of bees to guttation should not become a standard regulatory requirement for all substances. As guttation issues have been investigated with special focus for a few years only, the conclusions represent the current state of knowledge. However, more studies are required to evaluate the attractiveness of guttation fluid exuded from seed-treated plants. Further basic research on mechanisms of water collection of the bees and use of water in the hive are needed.

1. Identify crops for which it is not an issue (very little guttation and low concentration in guttation droplets), e.g. sugar beet

Some crops show guttation more frequently than others, and the intensity of guttation also varies. Whereas some crops show guttation only at younger growth stages, some may show guttation up to inflorescence.

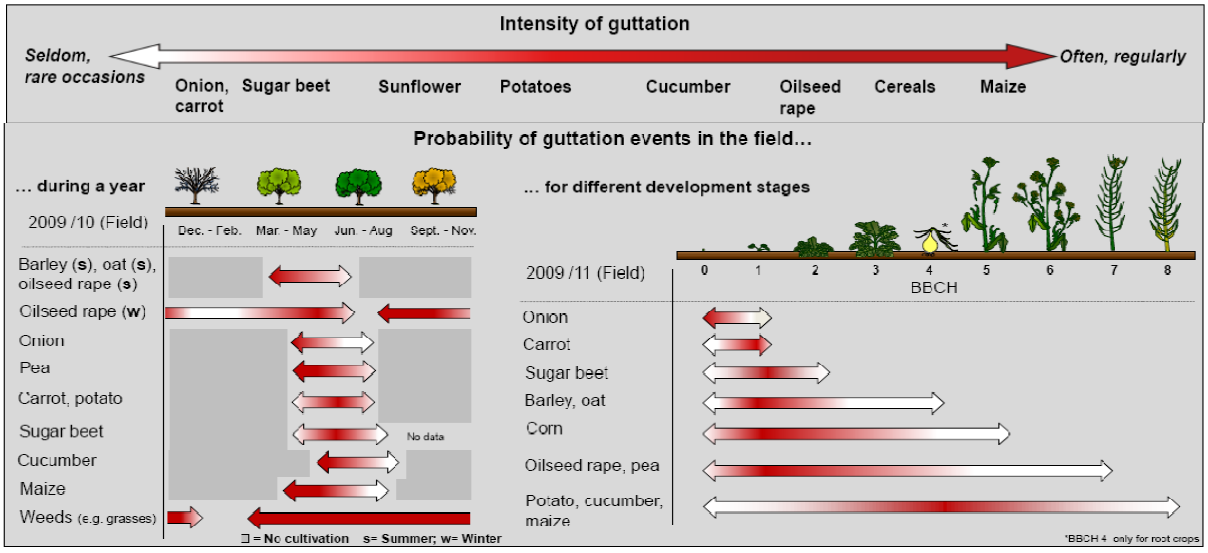

Figure H8: Intensity and frequency of guttation observed in field trials from Joachimsmeier et al. (2011).

Some crops show very low guttation probability and very small droplets, e.g. sugar beet; in the case of hops, no guttation is observed.

2. Identify conditions in which guttation does not happen

Guttation is reduced in very dry conditions. It is not yet possible to predict the climate conditions that trigger guttation; however, factors affecting guttation include soil humidity and air humidity.

3. Identify when bees use guttation droplets to drink

Very limited data are available concerning the portions of water used for different tasks in the hive (water needed for regulation of humidity and temperature of the hive and the brood nest (e.g. little droplets on larval cell walls for humidity, spreading of water on capped brood) and how much is used for drinking. In general, there are a larger number of variables, but it can be concluded that water uptake may occur during the whole year, even during overwintering. The water need of a colony increases with increasing brood rearing, and will be particularly high in spring, summer and early autumn.

4. Narrow down the situations in which guttation could pose a risk to bees

A potential water uptake of guttation droplets is mainly determined by the distance between colony and crop and the availability of other water sources. The risk of uptake of contaminated water is high if the colonies are located in closer proximity of the crop, but low with sufficient distance. If the crop is showing regular guttation activity and seeds are treated with systemic active ingredient with high intrinsic bee toxicity and findings of residue levels of high concern in guttation droplets occur, guttation could lead to a potential risk for bees.

However, since most studies were carried out in Italy and in Germany, more studies are required to evaluate the attractiveness of guttation fluid exuded from seed-treated plants in other countries where the local climatic conditions may enhance the risk of guttation for bees (e.g. Spain).

I. SPRAY APPLICATIONS

Exposure to molecules still present in the spray liquid

Exposure in the field

Spray drift is the transport process of pesticides in droplets generated during the spraying operation. These droplets are transported by the wind. The deposition of these droplets to a surface is mainly caused by gravity and, to a lesser extent, in the case of very small droplets, by turbulence. Spray drift during field spraying is influenced by a number of factors including applicator factors (droplet size, dose rate, boom height, etc.) and climatic conditions (wind speed, humidity, etc.).

The simplest approach to determine the exposure of bees to plant protection product molecules in spray liquid is to assume that the bees are exposed to the same mass per area as the agricultural field. To test this hypothesis, it is necessary to estimate the surface area of a bee (only from one side). Canton et al. (1991) give a value of 0.5 cm^2 for honey bees measured from a photograph. From the measurements of Cooper et al. (1985) we calculated a surface area of 1.9 cm^2. However, it is not clear from their description whether this is the area of one side only or the complete surface area. Johansen et al. (1983) report a total surface area of a honey bee worker of 1.86 cm^2. So it is likely that the 1.9 cm^2 reported by Cooper et al. (1985) is the total surface area. Tentatively we propose to assume 1 cm^2 as the surface area of one side of a honey bee forager and to use an uncertainty range of 0.5–2 cm^2 when this uncertainty is relevant.

Koch and Weisser (1997) studied the exposure of honey bees during pesticide application under field conditions. They applied a fluorescent tracer (sodium fluorescein) at a dose of 20 g/ha. Bees were collected at the closed hive entrance over a period of 20–30 minutes in 5-minute intervals. About 100 bees were collected at each sampling point. Each bee was rinsed with 10 mL of water to extract the tracer. Mean initial deposit per trial varied from 1.62 to 20.84 ng/bee (mean 6.3 ng/bee) in apple orchards (nine trials) and from 6.34 to 35.77 ng/bee (mean 18.2 ng/bee) in *Phacelia* crops (five trials) with a very few highly contaminated individuals (>45 ng/bee). Assuming a surface area of 1 cm^2 for a bee, the dose of 20 g/ha corresponds to 200 ng/bee (assuming 0.5 cm^2 would lead to 100 ng/bee). So the masses per bee measured by Koch and Weisser (1997) are considerably lower than the applied mass per surface area. Possibly the bees avoided being present in the spraying cloud or they intercepted less product due to interfering aspects (e.g. run-off or bouncing off from hydrophilic insect cuticle). It is also possible that the extraction efficiency of the tracer was less than 100 % because the bees took up part of the tracer (the extraction efficiency was not checked by the authors). However, this efficiency is unlikely to be much smaller than 100 % for such a water-soluble tracer.

Although Koch and Weisser (1997) conducted 14 experiments in 6 years, their studies do, of course, have limitations: they considered only (i) two crops (apple orchards and *Phacelia*), (ii) one type of spray equipment for each of these two crops (an axial fan sprayer and a 12-m boom sprayer), (iii) a fluorescent tracer and (iv) honey bees. The Panel therefore recommends that such experiments are carried out in different crops with different types of spray equipment considering different types of formulated products and considering also bumble bees. Koch and Weisser (1997) did not report the weather conditions during their experiments. The Panel recommends that weather data are also collected in future experiments.

EFSA (2008b) reports median and 90th percentile values of residue unit dose (RUD) of 17 and 54 mg/kg, respectively, for foliar-dwelling invertebrates. The RUD is defined as the mass of substance per mass of insect after spraying a dose of 1 kg/ha. Assuming a mass of a bee of 0.1 g, these values correspond to 42–108 ng/bee for a dose of 20 g/ha. These values are somewhat higher than the masses measured by Koch and Weisser (1997).

If honey bees would fly through the cloud of spray droplets, they could in principle catch much more plant protection product than the mass applied per 1 cm^2 of the field. However, in view of the studies by Koch and Weisser (1997) this is unlikely to occur. Therefore, the Panel proposes to assume as a conservative assumption that honey bees in the field during or shortly after spray applications are exposed to a mass corresponding to the mass sprayed to 1 cm^2 of the field.

No measurements are available on the spray deposition of bumble bees. In the absence of this, the Panel proposes following the same approach as for honey bees but using an estimate of the one-sided surface area for bumble bees.

These proposals for assessment of direct exposure to the spray cloud are only relevant in future risk assessment procedures that are more refined than the current approach. The current approach for honey bees is to perform the risk assessment resulting from the exposure to the spray solution on the basis of the HQ (Chapter 2). The HQ is defined as the dose in g/ha divided by the LD_{50} in µg/bee and the criterion is HQ < 50. This HQ has a strange unit (10^6/ha), which makes the trigger value of 50 difficult to grasp. If we assume that a bee has a surface area of 1 cm^2, a dose of 1 g/ha corresponds to 0.01 µg/bee. So if we redefine the HQ as the dimensionless quotient PEC/LD_{50}, where PEC is the predicted environmental exposure concentration, defined as mass per bee with unit µg/bee, then the trigger value for this new HQ would become 50 × 0.01 = 0.50 because the value of the numerator of the new HQ is 100 times lower than the value of the numerator of the old HQ. This new HQ can be seen as the inverse of a TER (LD_{50}/PEC). Thus, this TER would have a trigger value of 2, which is low compared with TER values used in lower tiers for other organisms. However, the study by Koch and Weisser (1997) suggests that that the true PEC is considerably lower than assumed in the

calculation of this TER. So a more realistic estimate of the PEC would lead to a TER of the order of magnitude of 10, which is closer to the TER for other types of organisms.

Exposure in the hive and in nests of bumble bees

Spray drift will of course also result in exposure of the hives at the edge of the field. Spray droplets will be deposited on the walls of the hive and some droplets may directly enter the hive via the air. Part of the spray solution attached to the bees exposed in the field will be deposited in the hive and thus contribute to a possible build-up of residue in the hive. These exposure routes are difficult to quantify realistically. A worst-case assumption could be to assume that each bee was exposed in the field to a mass deposited on 1 cm^2 and that this mass is completely deposited in the hive (which is of course unlikely). This would correspond to 10 µg of substance per bee at an application rate of 1 kg/ha. A pollen forager carries in the order of 100 mg pollen per day to a hive and in the order of 100 mg per day sugar in nectar (Table 3.1). Let us consider the first 10 days after an application of 1 kg/ha and assume that the nectar contains 50 % sugar. So in this period a bee carries in the order of 1 g of pollen and 2 g of nectar to the hive. Appendix G (Tables G7 and G10) shows that the concentrations in nectar and pollen for an application of 1 kg/ha are 0.1–12 mg/kg and 0.0002–150 mg/kg respectively. This corresponds to 0.2–170 µg of substance per bee. The 10 µg of substance per bee is in this range, so, for compounds with low concentrations in nectar and pollen, the mass entering the hive as spray solution may exceed the mass entering via pollen and nectar. Nests of social non-*Apis* bees in the soil may of course also be exposed by spray solution attached to bees entering the nest and the order of magnitude of the substance mass that may thus enter the nests under worst-case conditions may be estimated using an approach similar to that described above for the hives of the *Apis* bees.

Exposure to molecules present in soil, water and air

Exposure via the air in the field and in the hives

The volatilisation of pesticides from crops and fallow soil can be described by empirical relationships between the measured volatilisation of pesticides and their physicochemical properties. The relationship for volatilisation from fallow soil also includes the effect of soil properties. Smit et al. (1997, 1998) analysed available volatilisation measurements after spray applications and found that the volatilisation from crops can be correlated with the vapour pressure of the pesticide and that the volatilisation from fallow soil is a function of the fraction of the pesticide that is in the gas phase in the soil. The authors established empirical relationships between cumulative volatilisation and properties of the plant protection product molecule. For volatilisation from plant surfaces under field conditions they found for a limited dataset:

$$V_7 = 33.7\ P^{0.466} \qquad (3.1)$$

where V_7 is the percentage volatilised within 7 days after the application and P is the saturated vapour pressure in mPa at 20–25 °C. So, for a vapour pressure of 1 mPa, V is 33.7 %, and for 0.1 mPa it is 12 %. About 40 % of the plant protection products registered in the Netherlands have saturated vapour pressures above 0.1 mPa (personal communication A.M.A. van der Linden, 2011). So these findings indicate that a considerable fraction of the plant protection products may show significant volatilisation from plant surfaces. For volatilisation from soils under normal to moist field conditions Smit et al. (1997) found:

$$V_{21} = 71.9 + 11.6\ \log(100\ F) \qquad (3.2)$$

where V_{21} is the percentage volatilised within 21 days after the application and F is the fraction of the substance in the gas phase in the top layer of soil. The Panel used Eqn 3.2 to calculate the volatilisation for a substance with a molar mass of 200 g/mol and a water solubility of 100 mg/L as a

function of its saturated vapour pressure and organic matter/water distribution coefficient (K_{om}). This volatilisation was calculated using the soil properties of the scenario selected for concentration in the liquid phase in the central zone by EFSA (2012; see Table 3.4). It can be expected that this is a soil with comparatively high volatilisation rates because the concentration in the liquid phase is closely related to the concentration in the gas phase in soil. The results shown in Figure I1 indicate that the fraction volatilised from soil can be also in the order of 10 % for a saturated vapour pressure of 0.1 mPa. So these findings indicated that also significant volatilisation from soil surfaces may occur for a wide range of plant protection products.

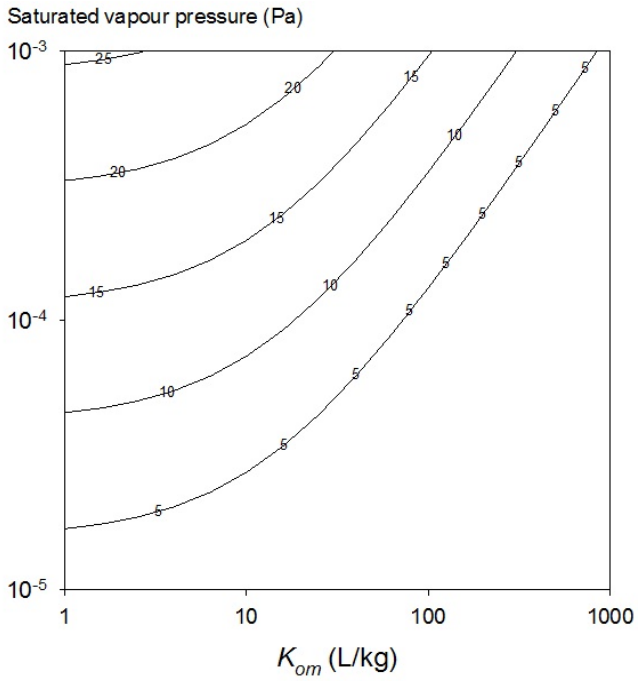

Figure I1: Contour diagram of the percentage of the dosage volatilised from bare soil as a function of the saturated vapour pressure and the K_{om} of the substance. The calculations were made with Eqn 3.2 for a substance with a molar mass of 200 g/mol and a water solubility of 100 mg/L and for a top layer of soil with an organic matter content of 1.8 %, a dry bulk density of 1.46 kg/L and a volume fraction of water of 0.347.

Volatilised molecules enter the air and are transported by atmospheric processes. The knowledge for calculating concentrations in the air from known sources of pollution is well established. This knowledge is also relevant for exposure of workers in the field, bystanders and residents. Currently scenarios for human exposure via the air are being developed in the EU R&D project BROWSE (see https://secure.fera.defra.gov.uk/browse/index.cfm). These scenarios are likely to be useful also for exposure of bees in the air in the field (both honey bees and social and solitary non-*Apis* bees) and also in the air in the hives (for the honey bees).

Like any other living being, bee colonies need air to survive. Southwick and Moritz (1987) estimated the tidal volume of a small colony (kept in small nucleus hive bodies ($15 \times 20 \times 30$ cm) with approximately 2 000 bees) as 0.42 L/min (25.2 L/h). In his book about honey bee ecology, Seeley (1985) compiled studies related to thermoregulation. He mentioned that the airflow created by fanning bees in order to maintain the colony's temperature is of. 2.2–3.6 m^3/h.

Molecules of plant protection products present in the air at the edge of a field will enter the hive, contaminating the internal environment of the colony. Then the exposure of brood to the product depends on its concentration in the air and whether or not cells are capped.

In conclusion, the Panel expects that scenarios for exposure of bees via the air in the field and in the hive at the edge of field will be developed relatively easily once the EU R&D BROWSE project is complete.

Exposure in the soil

Social non-*Apis* bees make nests in soil, and this may happen to be in an agricultural field in which a plant protection product has been applied. They may then be exposed to the product via the gas and liquid phases in soil (and possibly also via the solid phase). EFSA (2012b) developed a methodology for assessment of exposure of soil organisms to spray applications in annual crops grown on level surfaces under conventional or reduced tillage. EFSA (2012b) developed scenarios for the three regulatory zones for both the concentration in liquid phase and that in total soil based on a 90th percentile of the spatiotemporal statistical population of concentrations. The Panel considers it defensible to assume that the concentration in the gas phase is directly proportional to that in the liquid phase so the scenarios for the liquid phase can also be used for exposure in the gas phase. The exposure assessment was limited to the top 20 cm because concentrations in this top layer were expected to be best correlated with effects on soil organisms. Nests of social non-*Apis* bees may be at depths in soil ranging from 2.5 cm for *Nomadopsis* to 2.5 m for *Hesperapis* (Stephn et al., 1969). EFSA (2012b) used as endpoints concentrations averaged over the top 1, 2.5, 5, 10 and 20 cm of soil. The Panel considers an exposure assessment based on the average concentration in the top 5 cm a defensible starting point for the exposure assessment of nests of social non-*Apis* bees in soil. It should be noted that the methodology derived by EFSA (2012b) is limited to annual crops on level surfaces and so does not apply to permanent crops (e.g. orchards) or crops grown on ridges (e.g. potatoes).

Exposure via surface water

As described previously, bees need water for various purposes and they may collect this from puddles on the field and from ditches, streams or ponds. FOCUS (2001) developed a tiered approach for the assessment of exposure in ditches, streams or ponds at the EU level resulting from spray applications. This approach included development of scenarios for numerical models for some 10 locations across the EU. EFSA is considering reviewing the level of protection of these scenarios for the exposure of aquatic organisms. As no better alternative is available at this moment, the Panel recommends using these scenarios for assessment of concentrations in ditches, streams or ponds.

EFSA (2008b) developed a simplified conservative approach for estimating the concentration in puddles on the field based on the assumption that this concentration is equal to the concentration in the runoff as simulated in the FOCUS surface water scenarios mentioned above. EFSA (2008b) assumed in this simplified approach that the substance is completely mixed over 5 cm depth of a top soil with an organic carbon content of 2 % and a dry bulk density of 1.5 kg/L. However, it is not certain that this approach is more conservative than the FOCUS surface water scenarios because (i) three of the four FOCUS runoff scenarios have organic carbon contents of 0.6–1.2 %, i.e. considerably less than 2 %, and (ii) the concentration in the runoff in the PRZM model (used as part of these scenarios) is mainly determined by the pore water in the top 1 cm instead of the top 5 cm. On the other hand, the PRZM model assumes (i) that the substance immediately after the spray application is mixed over the top 4 cm of soil, leading to a concentration that decreases linearly to zero at 4 cm and (ii) that only a certain fraction of the substance in the pore water in the top layer is transferred to the runoff. However, the description of the parameterisation of this exchange of substance between runoff and pore water in the PRZM manual is difficult to understand (Eqn 6-42 at pp. 6-16 of Suarez (2005) indicates that the fraction of substance available for runoff increases with depth, whereas their Figure 6.3 at p. 6-17 shows that this fraction decreases with depth). A further factor that may lower runoff concentrations simulated in PRZM is that runoff is always preceded by infiltration of water, which will lead to leaching of substance from the top 1 cm. In view of the complexity of this problem, the Panel recommends that the conservativeness of the simple approach by EFSA (2008b) is checked by calculations with these FOCUS scenarios for a range of substances. In the meantime, the Panel

recommends that concentrations in puddle water are estimated directly from concentrations in runoff of the mentioned FOCUS scenarios in cases where this would be relevant.

J. SEED TREATMENTS

Recently, several bee mortalities have been reported during maize-sowing operations in numerous European countries. In northern Italy from 2000 to 2008, some spring bee incidents were linked with maize seed dressed with insecticides (Bortolotti et al., 2009). In 2008, over 700 beekeepers with around 12 000 hives in the Rhine Valley, Germany, were affected by insecticidal dust drift during sowing of maize and contamination of the nectar and pollen of neighbouring flowering crops (Pistorius et al., 2009), and similar incidents occurred in France, Austria and Slovenia (Alix et al., 2009a). Reports of bee killing coinciding with the peak period of maize planting were observed also in Indiana (USA) in spring 2010 (Krupke et al., 2012). All these incidents were linked to dust dispersed during maize-sowing operations. In fact, it was shown that pesticides may be dispersed from the pneumatic drilling machine during sowing (Greatti et al., 2003, 2006) and bees may come in contact with these contaminated dusts in several ways: by direct contact (when bees fly through the toxic cloud in the sown field), by indirect contact (when bees walk on contaminated leaves of the vegetation surrounding the sown field) or by ingestion (when bees collect nectar, pollen or dew from the vegetation contaminated with the dispersed dusts). Some of the pesticides used for maize seed dressing (e.g. clothianidin, imidacloprid, thiametoxam and fipronil) are extremely toxic to bees, with lethal and sublethal effects depending on the level of exposure.

Exposure during application

Seed treatments and granules are one of the most important formulations involved in so-called non-spray application (NSA). They can be applied broadcasted over the field (eventually followed by incorporation) and buried (precision application). Special cases are the so-called coated seeds.

Pesticide formulations for seed treatment are applied either as dry powders, slurries or liquids which are mechanically mixed with seeds. Dry powder formulations tend to sift off the seeds readily and can "drift" more easily. Liquid treatments are fixed better and are more difficult to remove from the seeds. Seed treatment can be achieved with special application equipment in seed company plants or on-farm in the planters' box. Generally speaking, they correspond to relatively low dosages, in the order of magnitude of 10–100 g/ha.

Treated seeds can be applied in different ways, which can have a different effect on the (dust) drift, runoff and leaching behaviour of the active ingredient: (1) broadcast (with or without incorporation into the soil) or (2) buried.

Figure J1: Schematic overview of exposure routes for non-plant exposures from treated seed applications.

The different routes of exposure are given in Figure J1 and are numbered as:

1. deposition of dust particles on the soil in the field;
2. dust deposition on puddles in the field;
3. dust particles intercepted by flying bees in the field and at the edge of the field;
4. dust drift out of the field in the air exchange system of the hive;
5. dust drift out of the field on the soil and the border plants.

Each of these routes can have a particular effect on several and different stages and types of bees. In the following sections they are discussed in detail.

The level of pesticide concentration to which bees can be exposed from treated seed during sowing operation is extremely variable and depends on the quality of the treated seeds (and also dust present in the bags), the machinery used, the modality of contact with the active ingredient, the time from the starting of sowing, the size of the sown area, the quality and quantity of vegetation in the margin of the field and the meteorological conditions.

Altogether dust drift emission is a complex situation, and exposure in off-crop areas depends on emission from the field and on type of vegetation where organisms are active and potentially exposed. The emission from the field being sown is influenced by:

- wind speed and direction;
- soil conditions (wet soil should reduce drift);
- the number of seeds used per hectare (more seeds result in more dust);
- the area sown (the wider sown opposite to the wind, the higher the emission);
- the abrasiveness of dust from seeds;

- the type of sowing machinery and the possible use of drift reduction devices (e.g. deflector);
- the content of active substance in dust (variable but usually higher with increasing dose).

For exposure assessment of dusts, recent research activities investigated the potential dust emission of different crops with different machinery. For an estimation of dust exposure for risk assessment purposes, it is important that quality criteria are defined and guaranteed and also machinery including devices for drift reduction, e.g. efficacy of deflectors is tested.

A detailed overview of the factors involved in potential dust exposure following sowing of different crops is given in the Appendices for the presence of free dust in the seed bags (Table J2), the Abrasion of treated seeds (Appendix K Table K2) during application (Tables J3–4), the residues in dusts (Tables J5 and J6) and machinery technique (Figure J2).

Table J2: Mean (Max) amount of *free dust from seed bags* of several crops sieved by the JKI (Heimbach et al, 2011b)

Crop	**Seeds (kg or no/ha)**	**Fine-grained (<0.5 mm) dust (maximum values) (g/ha)**	**Coarse-grained (>0.5 mm) dust (maximum values) (g/ha)**	***N***
Cereals 2009 Barley Wheat Rye	 180 kg 250 kg 150 kg	 11.3 (31) 9.5 (28) 5.1 (24)	 46.0 (116) 6.7 (19.2) 6.6 (32.9)	 30 31 23
Maize 2008 2009	 100 000	 4.5 (25.6) 1.99 (5.8)	 6.1 (47.3) 3.5 (12.1)	 82 45
Oilseed rape 2007 2008	 700 000	 0.81 (4.72) 0.27 (0.88)	 – –	 22 24
Sugarbeet	100 000	0.035 (0.125)	–	22

Presence of free dust in the seed bags:

Relatively high levels of dust can be present in the seed bags of different batches of cereal, maize and oilseed rape seed bags and in sugarbeet seedboxes taken from different seed treatment facilities and treated with different pesticide products. From treated maize and cereal batches quite high amounts of dust were sieved and calculated for a 1 ha sowing rate. Maize and cereals were quite heavily contaminated with dust: up to 31 g/ha and 116 g/ha for fine-grained dust (<0.5 mm) and coarse-grained dust (> 0.5 mm), respectively (Heimbach et al., 2011). Detailed results are given in Table J2. An improvement was visible from 2008 to 2009 in maize and oilseed rape (Heimbach and Stähler 2010a,b). Sugarbeet pills were quite clean, with very low amounts of dust, some of which originated from the opening of the boxes. Fine dust particles smaller than 0.5 mm, which are more likely to drift, were detected in all crops, whereas larger particles, originating from particles of the seeds, were found mainly in maize and cereals (Pistorius et al., 2009; Heimbach and Stähler, 2010c). Still not too much is understood on particle size of dust. The Heubach method selects only fine particles; large particles may even stay in the drum. Dust from some crops, such as cereals and maize, have also larger dust

particles, whereas in other crops only fine particles are released, which are more prone to drift. At present, it is expected that, overall, Heubach dust values may well represent emission into the field.

Seed preparation is considered to be one of the most critical steps as cleaning is widely recognised as a means to reduce dust values. Removing dusts before treatment improves the adherence of coating onto seeds. Coating quality, and, in particular, the presence of dust on the seed during the coating process, is known to contribute to dust release during the handling of seeds and seed bags (Heimbach and Stähler, 2010c). As both parameters, "amount of dust" and "a.s. in dust", directly influence the possible off-crop exposure, Heubach abrasion values might also be recalculated as "dust a.s./ha", taking into account both maximum values for abrasion as well as content of a.s. in dust.

Table J3: Heubach values of maize batches collected in Germany, France and Hungary (Heimbach, 2012)

Year	No of samples/Heubach test facility/sample country	Heubach in g/100 000 seeds	Max. Heubach-value
2008	53/Bayer CropScience, DE	1.11	4.15
2009	41/JKI, DE	0.30	0.56
2009	40/(LTZ), DE	0.53	0.91
2010	43/LTZ, DE	0.33	0.66
2011	34/LTZ, DE	0.18	0.40
2010	1737/Cruiser Monitoring in F	0.26	0.84 90 % < ca. 0.45
2011	2/JKI, HU	1.22	1.54

Abrasion of treated seeds during application: the Heubach–Dustmeter test method was introduced in 2008 and proposed for standardised measuring of dust abrasion. The Heubach method mainly detects fine dust particles, which are most prone to drifting. In Table J3, Heubach values for 2008, the year of the bee poisoning incidents in Germany, and for subsequent years following improvement of the situation are presented. It is clear that abrasion improved quite drastically after 2008 in Germany and some other countries (Heimbach, 2012). However, the table also shows that a change is possible only if all seed treatment facilities work correctly to avoid dust. The Heubach values for oilseed rape have improved within the last year. Among more than 200 different oilseed rape seed batches sampled in 2009, Heubach values were less than 0.5 g/700 000 seeds in more than 95 % of cases (Heimbach and Stähler, 2010a).

Table J4: Mean Heubach values (g/ha) of more than 300 batches of cereal seeds from several coating facilities sampled in 2008–2010 (calculated for maximum sowing densities in kg/ha) (Heimbach, 2011a)

Crop	kg/ha	2008	2009	2010	2010 min–max
Barley	180	3.0	2.6	1.9	0.37–4.51
Wheat	250	7.7	3.4	2.3	0.30–13.7
Triticale	170	–	4.1	0.9	0.44–1.39
Rye	150	6.3	0.7	1.0	0.31–3.11

Heubach values presented in Table J4 indicate that, besides maize, cereals seem to have most problems with dust abrasion. The use of adequate sticker during coating seems to be a very important improvement regarding dust abrasion. Fifty wheat samples from 2011 had a Heubach value of 3.5 g/250 kg seeds when no sticker was used compared with 61 batches of wheat of about 0.8 g using a sticker. Something similar was detected for barley seeds (Heimbach, 2011a). Altogether this means that the dust content of seed bags and abrasiveness of treated seeds can be improved drastically if adequate technique is used. However, it also show that seeds can be heavily contaminated varying more than 10-fold among different seed batches.

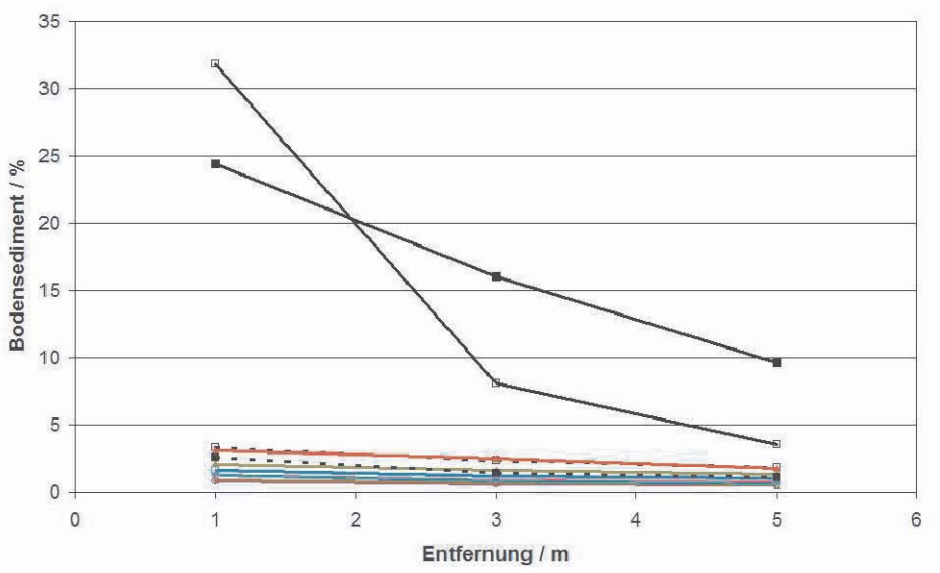

Figure J2: Dust drift reduction with deflectors. Dust deposition at different distances of modified machines (coloured lines) compared with standard machines (black lines).

The Julius Kühn-Institut tested a number of sowing machines and their accessory kits regarding the potential for dust emission during sowing (Rautmann et al., 2009). Compared with unmodified standard equipment, the drift of these models was at least 90 % lower.

The sowing machines used today are mostly precision airplanters with vacuum singling. In southern Germany, Monosem sowing machines are widespread, but Kuhn, Amazone and Gaspardo machines are also used. Together with the manufacturers, the JKI has established a drift test in which modified sowing machines are tested against standard machines with high drift. In autumn 2008, the sowing machines of all well-known manufacturers were tested and those which were shown to reduce drift by at least 90 % were registered in the JKI list as “drift reducing maize sowing machines”. In addition, methods for measuring direct drift when sowing corn seeds outdoors and to determine drift reduction

of corn single-grain sowing machines operating with suction air have been described. Precision airplanters for maize already used by farmers can be equipped with drift reduction kits tested and registered by the JKI (Rautmann et al., 2009).

Exposure routes from treated seed applications

One of the main conclusions of the EFSA Opinion on NSA (EFSA, 2004) is that broadcast application of treated seeds (with and without subsequent incorporation) is also considered to be a relevant route of dust exposure. Dust formation during application can eventually become a source of exposure to bee populations. In Appendix J, Figure J1, a schematic view of the possible routes of exposure is given. Whereas some crops, e.g. maize and cereals, may show considerable amounts of dust, some coated seeds, e.g. sugar beet pills, are considered as dust free although dust particles can be formed during application by abrasion.

The dust problem is recognised by the EU,[19] stating that appropriate measures are to be taken ("adequate seed drilling equipment shall be used to ensure a high degree of incorporation in soil, minimisation of spillage and minimisation of dust emission"). A new document (the EU GD on dusts) is currently under development and in discussion between the different participating countries. In this GD, next to the abrasion analysis methods, different machine/sowing techniques and also the active ingredient content of the dust of different crops is being considered. Unfortunately, it is not available yet. Recently, particular criteria are defined for dustiness of treated seeds in several Member States (Pistorius, personal communication).

Dust exposure occurs only during application of dry products, such as dusts, granules and treated seeds. Depending on the particle size, the dust particles can drift in a similar way to spray drift. Dust can be present in the treated seed preparation but can also be formed during application by abrasion. However, in comparison with spray droplets, the particles do not evaporate during transport. In addition, contact with the target surfaces may be less because there is no direct absorption at the surface, but, depending on hairiness of plants and plant structure, different plants or parts of plants may accumulate different amounts of dust.

As one of the tasks in the EFSA Opinion on NSA (EFSA, 2004), a method was proposed to estimate dust drift by modelling it in a similar way as done before for spray droplet drift. As compared to droplet drift, experimental data of dust drift during application of treated seeds were scarce (EFSA 2004) but several datasets are available. Dust drift of very small particles, can behave in a similar way as vapour drift because dust drift can also be influenced by air turbulence effects due to the very low gravity effects of small dust particles. Still, this methodology, originally intended for surface water deposition, can be proposed as starting point for dust deposition on soil. As dusts may be intercepted by neighbouring plants and flowers, a correction factor may be needed for estimation of these residues.

Dust deposition on soil surface in the field

Primary exposure to soil deposition in the field is especially important for social non-*Apis* bees and solitary bees because they nest in soil and thus dermal exposure can be expected.

The worst-case soil deposition in the field can be estimated when no drift of the dust is occurring and when the maximum allowable amount of dust is considered. There is also no need to consider interception factors by plants in-field because seed applications are done on a bare soil surface.

[19] Commission Directive 2010/21/EU of 12 March 2010 amending Appendice I to Council Directive 91/414/EEC as regards the specific provisions relating to clothianidin, thiamethoxam, fipronil and imidacloprid, Official Journal of the European Union L 65/27.http://eur-lex.europa.eu/GGTSPU-styx2.bba.de-20618-1112068-VxEfiu2st5oivCKS-DAT/LexUriServ/LexUriServ.do?uri=OJ:L:2010:065:FULL:EN.PDF

As a very conservative approach, the maximum dust amount per hectare may be estimated using 10 % of the maximum rate of active ingredient on the seeds/ha as first tier for soil exposure and the aquatic environment. The value of 10 % was never exceeded in the experimental data. Based on these data, including a conservative filtering capacity correction factors of neighbouring crops (e.g. 10×), the full rate per hectare for drift into neighbouring crops may be used. This first tier approach can be replaced by experimental data of dust deposition during application of treated seeds in the field, but these data are scarce for the moment. More information on dust deposition on soil surface measured with Petri dishes is presented in Figures J3–7 and Table J5.

Therefore, it is recommended that preliminary (literature search) study be carried out in order to confirm and validate the previous approach. For the behaviour and fate of these deposits on and in the soil, the comments provided in the sections on "Exposure in soil" and "Exposure via surface water" should be taken into consideration.

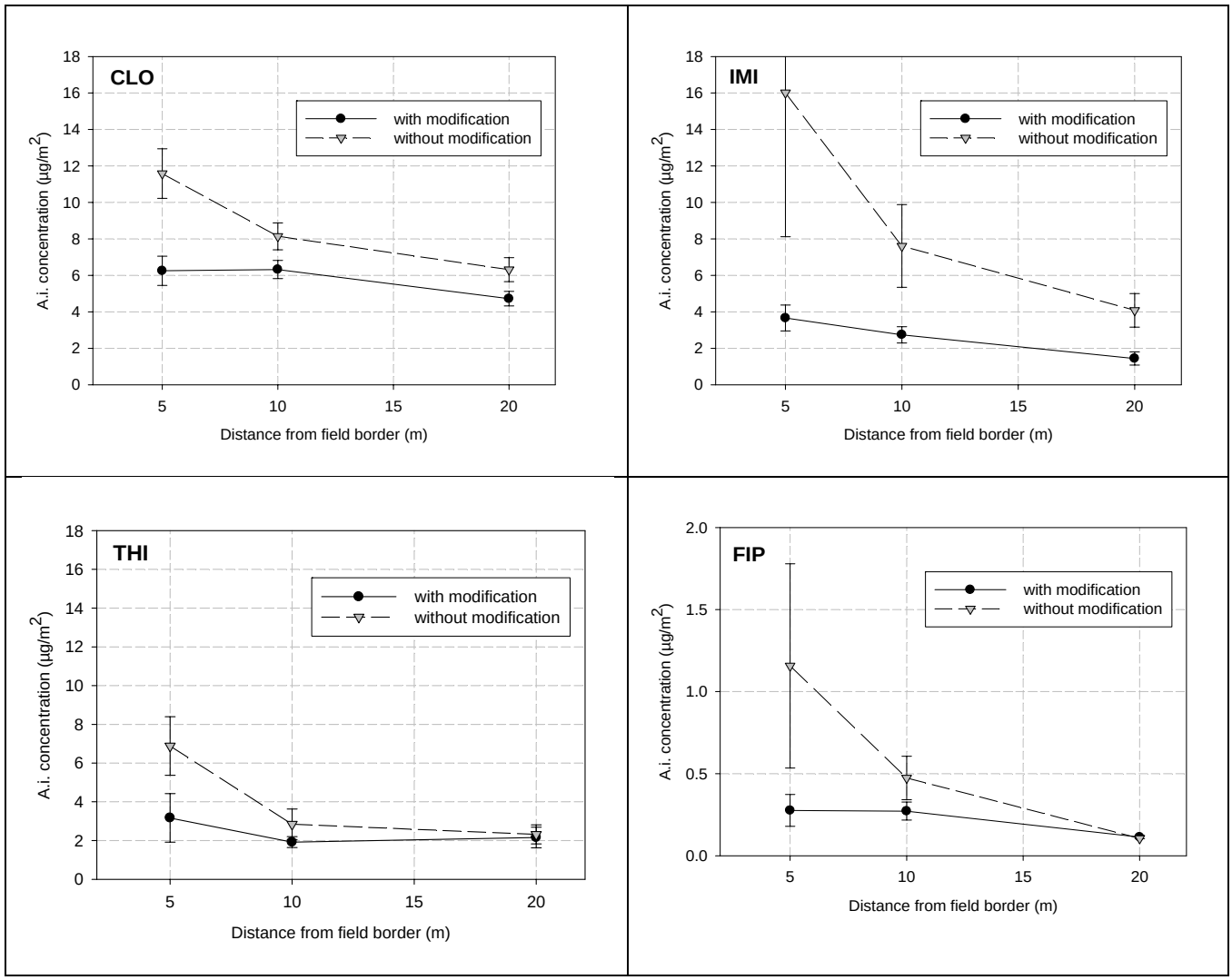

Figure J3: Results of field trials: mean values and corresponding standard errors of concentrations for clothianidin (CLO), imidacloprid (IMI), thiametoxam (THI) and fipronil (FIP), at the three sowing distances (from CRA-API, 2010). A Gaspardo Magica six row-precision pneumatic seeder (75 000 seeds/ha) with (modified) and without (unmodified) deflector was used. The seeds (hybrid employed PR32G44; Pioneer Hi-bred, Johnston) were supplied by A.I.S. (Italian Seed Association) in 2010. The quantity of dust abrasion was measured with the Heubach test and was under 2 g/q.

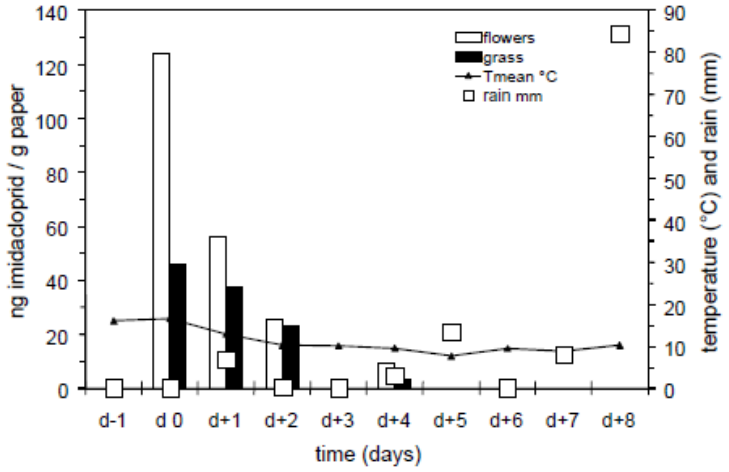

Figure J4: Residues of imidacloprid found in flower and grass samples collected near the field sown. Rain and temperature registered during the sowing period (d0: sowing day) from Greatti et al. (2006)

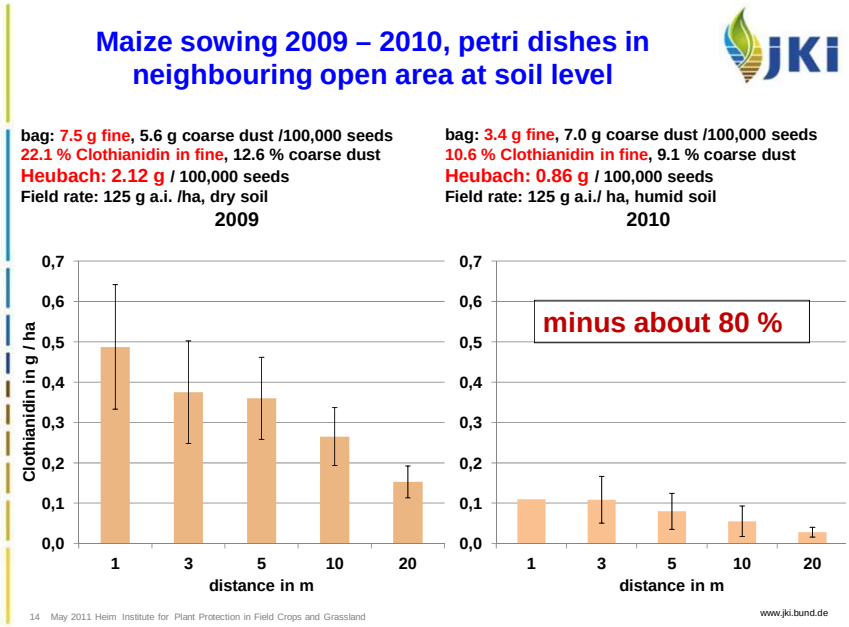

Figure J5: Residues in Petri dishes at 1–20 m distance from the drilling area for sowing maize with deflectors (90 % drift reduction) and seed batches containing 469 mg clothianidin/100 000 seeds on Heubach filter in 2009 and 91 mg in 2010 (Heimbach, 2012).

Table J5: Experiments on drift (all seed batches treated with clothianidin) and residues of clothianidin in the crop area, in Petri dishes, at 1, 3 and 5 m from the sowing area (see Heimbach et al., unpublished).

Year of drift	Treatment a.i.	Heubach value (g)	% a.s.	Heubach	Mean residue in Petri dishes

experiment			dust	a.s./ha	at 1–5 m distance (g a.s.)
Maize 2009	1.25 mg/seed	2.12 g/100 000 seeds	22.1	0.469 g	0.41
Maize 2010	1.25 mg/seed	0.86 g/100 000 seeds	10.6	0.091 g	0.10
Maize 2011	0.5 mg/seed	0.45 g/100 000 seeds	19.1	0.086 g	0.15
Rape 2011	10 g/kg	0.38 g/700 000 seeds	6.3	0.024 g	0.021

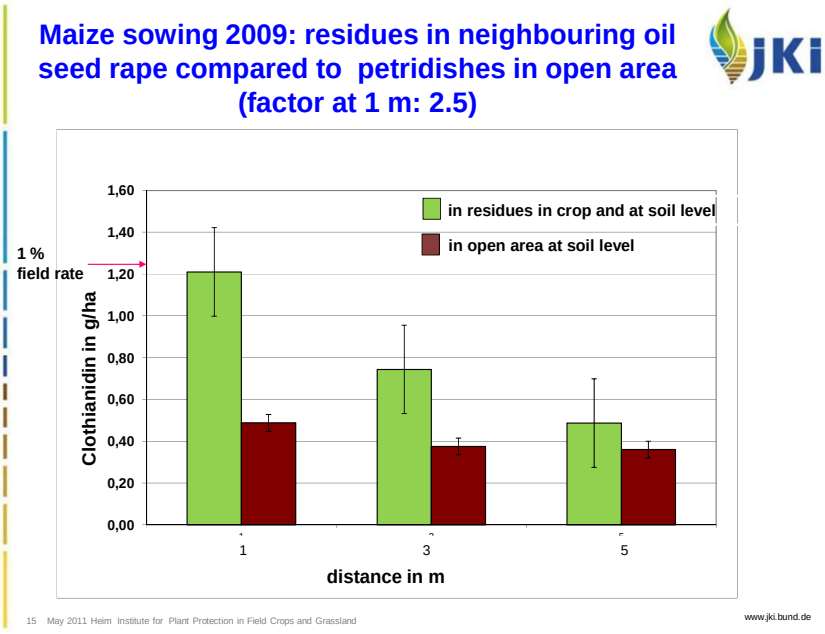

Figure J6: Residues of clothianidin detected in Petri dishes (on bare soil in the crop) and flowering off-crop plants at different distances from the sowing area of clothianidin-treated maize seeds directly after sowing in 2009 (Heimbach et al., 2011).

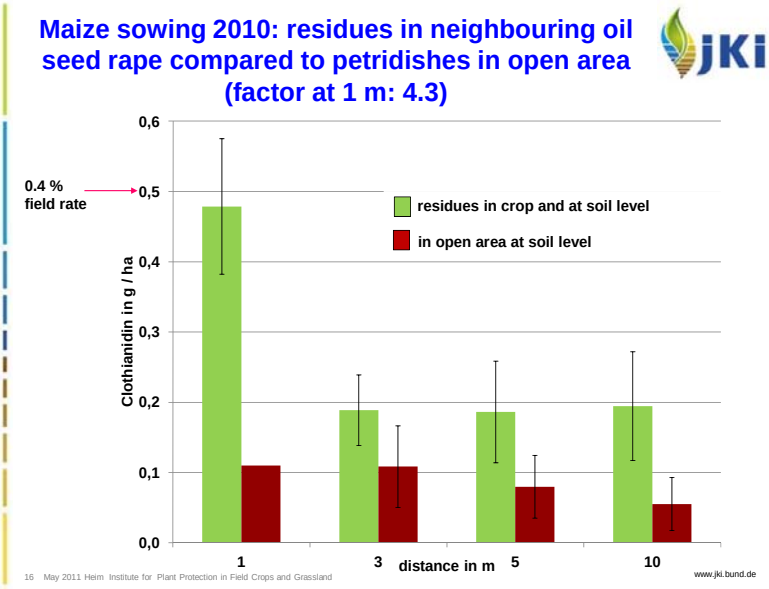

Figure J7: Residues of clothianidin detected in Petri dishes (on bare soil in the crop) and flowering off-crop plants at different distances from the sowing area of clothianidin-treated maize seeds directly after sowing in 2010 (Heimbach et al., 2011).

Dust deposition in puddles in the field: Dust formed during the application of treated seeds can also occasionally reach puddles in the field, which are possible drinking water sources for (forager) bees. The water can also be collected and used as a "cooling" system in the hive by its evaporation. The maximum concentration of the active ingredient in puddle water (g a.i./L) can be calculated by taking into account the maximum deposition per unit surface area of the puddle (similar to the calculation of the soil deposition in previous section), the surface of a standard puddle (m^2) and the volume of a standard puddle (m^3). No experimental data could be found in literature concerning pesticide concentrations in field puddles after treated seed applications. There is also a calculation method for estimation of pesticide deposits in puddles in the birds and mammals opinion (EFSA, 2008b).

Dust particles intercepted by flying bees in the field and at the edge of the field: Exposure may also occur during sowing when the bees are flying in the field to reach a foraging site. In principle, it is possible to estimate the amount of dust intercepted by flying bees by taking into account the maximum concentration of the cloud, flight speed and flight time and the body interception of a bee. Although this concept can be considered as a first tier approach, a lot of information on all these aspects is still needed. Recent studies confirm the presence of high insecticide concentrations in dust particles emitted during sowing of treated corn seeds (Pistorius et al., 2009; Marzaro et al., 2011; Girolami et al., 2012). Small particles (PM10), in particular, are characterised by high atmospheric mobility and can be efficiently intercepted by flying bees. Experimental determinations of total suspended matter (TSP) and PM10 concentrations at the field margin clearly indicated the presence of non-negligible levels of (even very small) particles (Tapparo et al., 2012). Even TSP values of up to 13.1 µg/m³ and PM10 values of 1.2 µg/m³ are observed at a distance of 10 m from the drilling machine during sowing of treated corn seed. Experimental matter emission measurements near the waste pipe of drilling machine during the sowing of treated corn seeds from 2009 and 2010 revealed emission of 0.46–1.53 g/ha, corresponding to 0.55–1.84 %, respectively, of the released amount of the active ingredient (Tapparo et al., 2012), although it should be recognised that the actual level of dust present was not reported and these data were generated prior to the Commission Directive on seed treatments. First tier values can eventually be compared with and replaced by experimental data. An interesting study was performed in a maize field (Girolami et al., 2011; Marzaro et al., 2011). Bees came in direct contact with dust dispersed into the air from the pneumatic sowing machine and it was shown that bees can intercept during flight more than 500 ng of active ingredient). In Marzaro et al. (2011), the caged bees

were forcibly exposed for half an hour to the emission (worst-case scenario) from the drilling machine. In Girolami et al. (2011), bees were conditioned to visit a dispenser of sugar solution whilst a drilling machine was sowing maize along their flight path. Samples of bees were captured on the dispenser, caged and held in the laboratory. The quantity of insecticide found in bee samples is summarised in Table J6. Also, in trials by the JKI (Pistorius, unpublished), wire cages were set up to measure the residues to which confined bees would be exposed during contact exposure at different distances to the field edge (Table J7).

Table J6: Quantity of insecticide (clothianidin and imidaclorpid) found in honey bee samples after exposure to the dust emission of the drilling machine. A Monosem NG Plus (Monosem, Largeasse-France) without deflector was used in all experiments. The seeds (hybrid employed X1180D 964890 in 2009 and PR32G44 in 2010; Pioneer Hi-bred, Johnston) were supplied by A.I.S. (Italian Seed Association). The 2009 and 2010 seed batches have a quantity of dust abrasion under the limit of 3 g/q. The quantity was tested with the Heubach test, considered the method which best allows standardisation of dust abrasion measurements within the seed industry (CRA-API, 2009; Nikolakis et al., 2009)

Insecticide	Conditions during exposure	Conditions after exposure	Quantity of a.i. (ng/bee)	Source
Clothianidin, 2010	Bees in cages exposed for 30 minutes to dust	Bees held in laboratory at high humidity	279 ± 142	Marzaro et al. (2011)
Clothianidin, 2010	Bees in cages exposure for 30 minutes to dust	Bees held in laboratory at laboratory humidity	514.25 ± 174.7	Marzaro et al. (2011)
Clothianidin, 2009	Bees in flight near the drilling machine	Bees captured at the dispenser 30 minutes after the start of sowing and dead in laboratory in high humidity	674	Girolami et al. (2011)
Clothianidin, 2009	Bees in flight near the drilling machine	Bees found dead on the ground in front of the apiary 3 hours after the start of sowing	161	Girolami et al. (2011)
Clothianidin, 2009	Bees in flight near the drilling machine	Bees found dead on the ground in front of the apiary the day after the start of sowing	118	Girolami et al. (2011)
Imidacloprid, 2009	Bees in flight near the drilling machine	Bees found dead on the ground near the dispenser 30 minutes from the starting of sowing	3661	Girolami et al. (2011)
Imidacloprid, 2009	Bees in flight near the drilling machine	Bees captured at the dispenser 45 minutes after the start of sowing and dead in laboratory in high humidity	442	Girolami et al. (2011)
Imidacloprid, 2009	Bees in flight near the drilling machine	Bees found dead on the ground in front of the apiary 3 hours after the start of sowing	500	Girolami et al. (2011)
Imidacloprid,	Bees in flight near the	Bees found dead on	53	Girolami et al. (2011)

2009	drilling machine	the ground in front of the apiary 4 h from the starting of sowing		
Imidacloprid, 2009	Bees in flight near the drilling machine	Bees found dead the day after the start of sowing	29	Girolami et al. (2011)
Clothianidin, 2009	Bees in flight near the drilling machine	Bees found dead at the sugar dispenser	396 (external deposit) 674 (total concentration)	Tapparo et al. (2012)
Clothianidin, 2009	Bees in flight near the drilling machine	Bees found dead at the hive entrance	155 (3 hours after sowing), 119 (24 hours after sowing)	Tapparo et al. (2012)
Imidacloprid, 2009	Bees in flight near the drilling machine	Bees found dead at the hive entrance	>3 000 (external, after sowing) 240 (external, 2 hours after sowing) <LOD (external, 24 hours after sowing) 3650 (total, at the end of the sowing) 325 (total, 2 hours after sowing) <LOD (total, 24 hours after sowing)	Tapparo et al. (2012)

Table J7: Residue analysis of bees in wire cages, sowing of maize 2011 (three repetitions on the downwind side (T1–T3), one on the upwind side (C) and one at a remote location (R)), no dust exposure

Distance	T1 (ng/bee)	T2 (ng/bee)	T3 (ng/bee)	C (ng/bee)	R (ng/bee)
1	5.7	10.1	9.3	12.8	0
3	4.2	5.2	5.1	0.1	0
5	4.1	3.8	3.0	0.0	Not available
10	2.1	2.7	3.0	0.0	Not available
20	2.2	1.8	2.3	0.0	Not available

Dust drift out of the field in the air exchange system of the hive near the field margin: Dust can enter the hive by convection because of the high airflows needed to aerate and cool the hive. The effect of the presence of pesticide residues on dust is here essentially a drift phenomenon. This problem is the same as off-field dust drift to soil and leaf surfaces in the field border (as discussed in the next section). In order to estimate the amount of dust entering into the hive, information is needed on how much air is needed for the thermoregulation in the hive. Southwick and Moritz (1987) estimated the tidal volume of a small colony (kept in small nucleus hive bodies (15 × 20 × 30 cm) with

approximately 2 000 bees) as 0.42 L/min (25.2 L/h). Seeley (1985) mentions that the airflow created by fanning bees in order to maintain the colony's temperature is 2.2–3.6 m^3/h.

Dust drift off-field on the soil and the border plants: Dust deposition on nectar, honey dew, pollen or dew from the vegetation contaminated with the dispersed dust may be an important exposure source for forager bees; bees may also be exposed on (soil) surfaces in the field.

Experimental data indicate that the active ingredient deposited on the vegetation surrounding the field can persist for several days after sowing. Greatti et al. (2006) found residues of imidacloprid in grass and flowers at least 4 days after sowing (Figure J4). High levels of residues were found in flowers, pollen and bee bread after the 2008 poisoning incidents following dust drift (Pistorius et al., 2009).

Dust drift experiments have shown that active substance originating from the drilling process can also be detected in off-crop areas after sowing of most seed-treated crops. Petri dishes with a wet filter paper inlay are frequently used for sampling of dust drift. The amount of active ingredient deposited on the ground during mechanical or pneumatic sowing at distances of 5, 10 and 20 m from the edge of the field has been measured in research projects (CRA-API, 2009, 2010; Heimbach and Stähler 2010c)

It seems that Petri dishes may represent a realistic scenario to evaluate the exposure in aquatic systems, for soil organisms and also bee exposure on soil or leaf surface. Exposure to dust present on the soil or leaf surface (expressed as g a.i./m² surface) is potentially relevant as an exposure route for resting foragers and bees which use leaves as nesting material.

For spray droplets, a lot of information on interception factors by plants is known (FOCUS, 2002; Van Beinum and Beulke, 2010), but this is not the case for dust interception. Very little is known about the interfering influences, and more studies are needed to determine the relations between the plant structure and the dust interception capacity of different leaves, which can depend on several factors, e.g. leaf structure, presence of hairs, presence of wax on leaf surface and canopy structure.

In some trials measuring dust drift, in addition to residues in petri dishes on bare soil also residues in directly neighbouring fields were analysed. Heimbach et al. (2010) measured residues in 2 different fields -bare soil (collection of residues in petri dishes) and oilseed rape (measurement of residues in plants)- adjacent to a maize field where a sowing operation had taken place. They found 2.5 times more active substance in 1 m distance in oilseed rape compared to petri dishes on bare soil, thus indicating a filtering capacity of directly neighbouring crops (Appendix K Figure K.1). The increase in 2010 at 1 m distance was a factor of 4.3. Residues in flowering oilseed rape directly bordering the sowing area were even higher with about 1 g/ha clothianidin in crop plants collected from 0–30 cm distance compared with only about 0.1 g in Petri dishes at 1 m distance (Heimbach et al., 2011). Thus, the use of Petri dishes to detect dust drift needs to be corrected for neighbouring plants. Different detection methods were used for dust drift measurement. The values of Petri dishes being filled with different wet carriers did not differ relevantly. A simpler method than using neighbouring crops is to use vertical exposed gauze nets, which compared with Petri dish values are up to about 10 times higher and which therefore might represent exposure in a neighbouring crop (Heimbach et al., 2011). However, more research on data linking residues measured in Petri dishes and flowers with residues in nectar and pollen following dust exposure is needed.

Exposure after application

Secondary exposure to dust after application of treated seeds can occur via several routes:

- evaporation of pesticides from deposited dust particles and subsequent vapour drift, although this is very limited because the active compound has low vapour pressure;
- off-field transport (with the wind) of deposited dust particles out of the field margin followed by redeposition;

- redistribution of plant deposits to the soil and puddles by wash-off from the plant surfaces.

Nevertheless these secondary exposure routes may be considered to be of less importance when compared with the primary exposure routes during seed application. It must be noted that the exposure of the forager bees by direct contact to plant deposits is considered in the section on plant exposure routes (see section 3.2). Secondary drift after the drilling process has been finished was noted in all of the JKI experiments only at very low rates compared with rates collected during the drilling process. For these measurements, new Petri dishes were exposed after drilling for secondary drift measurements and this proves secondary drift plays only a minor role compared to drift occurring during the drilling process itself.

Another method of exposure occurs when bees forage on follow-up flowering crops (e.g. oilseed rape) grown in a field where in past years dressed seeds were sown (e.g. maize, cereals). Thus, nectar and pollen may contain active ingredient taken by the plant from the soil. In fact, neonicotinoids may be extremely persistent in the soil; the half-lives of these compounds in aerobic soil condition can vary widely depending on the soil and climate, but are best measured in months (US EPA, 2003). Krupke et al. (2012) also demonstrated that clothianidin is present on the surface soil of agricultural fields long after treated seed has been planted in that field. All collected soil samples contained clothianidin (from 2.1 to 9.6 ppb), even in cases where no treated seed had been planted for two growing seasons. It is therefore important to take into account when the treated crop is not considered bee-attractive and the follow-on crop is highly attractive to bees.

K. INJECTION OF SOIL FUMIGANTS

Soil fumigants such as 1,3-dichloropropene and metam-sodium are injected as liquids into the soil at application rates in the order of 100 kg/ha at depths ranging from 15 to 20 cm. Metam-sodium in the soil is quickly transformed into methylisothiocyanate, which is the active ingredient. Methylisothiocyanate and 1,3-dichloropropene have saturated vapour pressures in the order of 1 000 Pa at 25 °C and move through the soil mainly via diffusion in the gas phase.

After application, the soil may be covered with a plastic foil to reduce the volatilisation into the atmosphere. The soil may also be refined and compressed by rolling to reduce this volatilisation. Application is at least 1 month before planting/sowing of the crop. Soil disinfestations need a soil that is not too wet and too cold and are thus not performed during winter and early spring. Treatments in greenhouses (not considered in this opinion) can be done over a more extended period.

Decades ago, computer models were developed and tested for simulating the behaviour of soil fumigants in soil and their volatilisation into the atmosphere (e.g. Leistra, 1971; Leistra and Frissel, 1975; Van den Berg, 1992, 1993; Van den Berg et al., 1999). It can be expected that a few to several tens of per cents of the dosage volatilises into the atmosphere. In view of the high dosages of soil fumigants, this will lead to comparatively high concentrations of these substances in the air above and around the field of application. Therefore, all types of bees are exposed to these substances via the air. Also the hives of the honey bees will be exposed via the air (if they are present at the edge of field at the application time). Also for these substances, the Panel expects that scenarios for exposure of bees in the air in the field and in the hive at the edge of field can be developed relatively easily once the EU R&D BROWSE project has been finished (https://secure.fera.defra.gov.uk/browse/index.cfm).

Exposure via water in puddles or via surface water is unlikely to be a major issue for soil fumigants that are injected into the soil if the field is covered with a plastic foil in the first weeks after application. This prevents exposure via water in puddles and leaching through drainpipes of these substances to a large extent because they dissipate from soil to a large extent in the first few months after application. However, if the soil is not covered with plastic foil, exposure via water in puddles and leaching through drainpipes may occur. This could be assessed by scenario calculations.

If nests of social non-*Apis* bees in soil in the field are present at the application time of the soil fumigant, they will be exposed to very high concentrations of this fumigant in the gas phase in soil (in view of the dosage in the order of 100 kg/ha). Exposure scenarios could, if necessary, be developed for such applications with comparatively little effort. However, it is unlikely that nests present above the injection depth will survive the mechanical disturbance of the soil that is caused by the injection equipment.

Table K1: Clothianidin residues (in %) in dust (a: fine dust, b: coarse dust) sieved from 50 different seed batches of maize treated with Poncho or Poncho Pro. SD, standard deviation. From Pistorius et al. (2009)

		dust < 0.5 mm Clothianidin		**dust > 0.5 mm Clothianidin**	
Clothianidin/kernel		**%**	**SD**	**%**	**SD**
N = 20	0.5 mg (Poncho)	18.5 a, A	5,7	11.4 a,B	3.5
N = 30	1.25 mg (Poncho Pro)	28.2 b, A	8,6	14.7 b, B	5.1
a: fine dust < 0.5 mm in g					
N = 12	1.2–1.75	23.8	6.4	16.2	6.6
N = 15	1.9–3.0	27.0	11.4	12.7	3.6
N = 23	> 3	23.2	7.8	12.9	3.7
b: coarse dust > 0.5 mm in g					
N = 17	0.5–2.7	27.9	6.4	15.5	5.5
N = 15	2.7–4.7	24.8	10.8	11.8	4.8
N = 18	> 4.7	20.4	8.0	12.7	3.3

t-test, raw data arcsin transformed (significant differences $p < 0.02$; a,b vertical, A, B horizontal).

Residues of active substance in dust: The percentage of active substance in dust is also a key factor that needs consideration. Regarding maize, clothianidin was detected in high percentage (Table K1, Pistorius et al., 2009), but varying between batches and in higher concentration in case of higher treatment rate. But some batches of seeds treated with 1.25 mg/seed had a lower residue content than others treated with only 0.5 mg, indicating large variability between seed batches. Lower residues in coarse dust can be explained by more plant material in this dust fraction which dilutes the percentage of clothianidin. In oilseed rape the mean concentration in fine dust was 3.4 % in the case of neonicotinoid treatment (rate up to 10 g a.s./kg of seeds) in 2008 and 5.9 % in 2009. But other ways of treatment, such as for carbosulfan, resulted in a higher concentration (mean 23.4 %) (Heimbach and Stähler, 2010a). In cereals, lower rates were detected for most fungicides being treated. Depending on the active substance and the seed dressing rate, residues between less than 1 % and up to about 5 % were detected in fine dust (Heimbach and Stähler, 2010b).

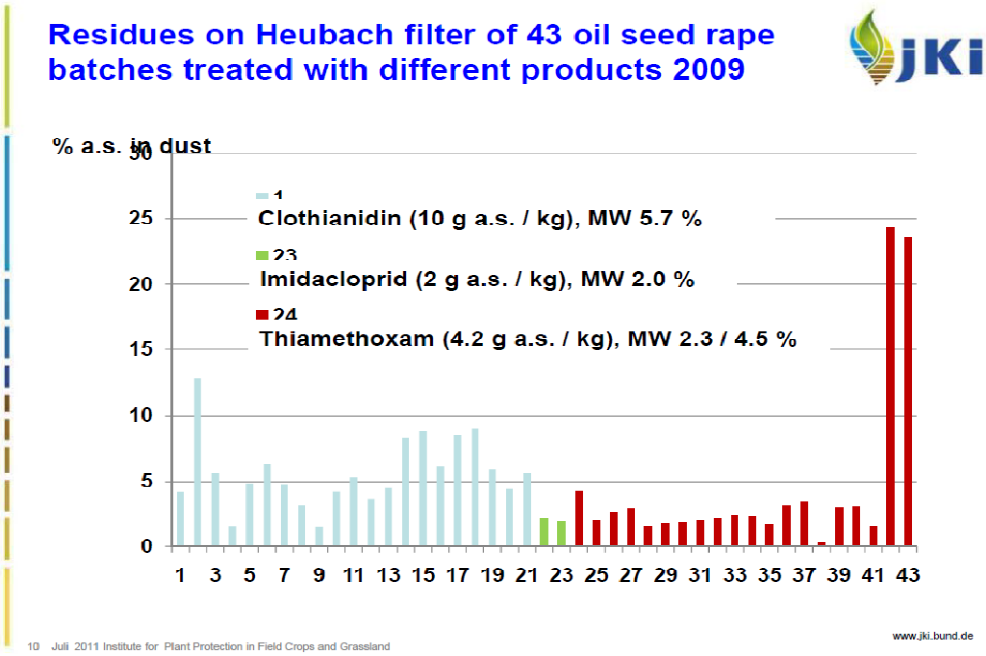

Figure K1: Percentages of residues of neonicotinoids in Heubach filter dust of treated oilseed rape in 2009 in Germany. (Heimbach and Stähler, 2010d)

The content of active substance in Heubach filter dust (Figure K1) can vary greatly even when the same amount of active substance per seed is applied. Coating recipes will influence not only the abraisiveness but also content of active substance in dust. Improvements in abrasion resistance can be achieved by improved seed treatment recipes. Concentrations of active substance vary between recipes and facilities. In addition to dust avoidance activities, only seed treatment recipes that minimise the content of active substance in dust should be used. Before new recipes are introduced for seed treatment, it is necessary to show that the content of active substance in dust is not increased. As the residue content in dusts may be highly variable, residue analyses of the dusts are needed. As both parameters, "amount of dust" and "a.s. in dust", directly influence the possible off-crop exposure, Heubach abrasion values might also be recalculated as "dust a.s./ha", taking into account both maximum values for abrasion as well as content of active substance in dust. Resistance of seeds to abrasion and loads of free dust within seed bags can be effectively reduced.

Table K2: Dustiness of seed coated with the four active ingredients, as measured with the Heubach cylinder (from CRA-API, 2010)

Name of the commercial product (active ingredient)	Data supplied by manufacturer		Data detected by CRA-ING		
	Fine dust (Heubach filter) g/q	Acive ingredient dose. mg/seed	**Fine dust. (Heubach filter) g/q**	Coarse dust. g/q	Total dust. g/q
Gaucho (imidacloprid) + Celest	**1.100**	1.000	**0.875**	10.83	11.71
Poncho (clothianidin) + Celest	**2.430**	1.250	**1.833**	19.16	20.99
Cruiser (thiamethoxam) + Celest	**1.200**	0.600	**0.950**	5.00	5.95
Regent (fipronil) + Celest	**1.780**	0.500	**0.723**	9.08	9.81

L. ASSESSMENT OF HONEY BEES' EXPOSURE

The results of the ranking are presented in Tables L1–6, M1 and N1, whether they are related to oral exposures from sprayed and non-sprayed (systemic and non-systemic) insecticides (Table L1), to exposures by contact and inhalation from sprayed and non-sprayed systemic and non-systemic insecticides (Table L2), to oral exposures from dusts of systemic and non-systemic insecticides (Table L3), to exposures by contact and inhalation from dusts from systemic and non-systemic insecticides (Table L4), to oral exposure from soil fumigant (Table L5) and to exposures by contact and inhalation from soil fumigant (Table L6). Results on honey bees are presented in Tables L1–6, results on bumble bees are presented in Table M1 and results on solitary bees in Table N1.

- Table L1: for nectar from plants, only nectar foragers were exposed and scored "4" given the amount of nectar ingested to achieve long-distance flights. For nectar stored in the hive, based on food intake, foragers were the most exposed. However, they were assumed to be less exposed to honey from nectar because the concentration of residues found in nectar is lower than in honey. Following the same reasoning, the other categories of bees were scored below "4", except winter bees which, towards the end of winter, feed exclusively on honey to thermoregulate and to feed the new brood. For oral exposures to pollen and bee bread, based on food intake data, larvae are the most exposed, followed by nurse/brood-attending bees. All exposures related to honey dew (from plants, stored in hive or ripen in honey) were considered to be equivalent to exposures for all types of nectar (from plants, in hive and honey) but during a shorter time window. All exposures related to water (from leaves, axils, puddles and surface) could not be fully evaluated given the unknown respective amounts taken by bees and therefore were scored "1–4?". However, given the known high levels of residues in water from droplets on leaves, water foragers exposed to this type of water were scored higher, i.e. "2", than water foragers exposed to the other types of water matrices.

- Table L2: exposures to propolis by contact were considered the most relevant for foragers because of the amount they can collect. However, given that this amount is highly variable in time (i.e. uncertainty on how much and how often foragers were exposed to propolis), a score of "3" was given. The exposure to larvae may be high if we consider that propolis may be in contact with larvae during their development but, thus far, it has not been quantified. Given the high amounts of residues found in wax and given that larvae are the most exposed to wax, this category of bees was scored "4". For honey bees, exposure to soil was considered minor whereas contact exposure to foliar residues was considered highly relevant, but lower than oral exposure to droplets. For that reason, foragers' exposure to foliar residues was scored "3" and interception to droplets "4". In addition, queens, drones and swarms exposures to droplets interception were considered very highly relevant and therefore scored "4" as well. Contact exposure to pollen was scored "3" for foragers because it was considered lower than exposure to droplet interception. The same exposure scores to pollen were given to nurse bees, which are the most exposed in-hive bees to this type of product. For the same reasons as described for droplet interception, exposure by inhalation of foragers, drones, queens and swarms was considered also very highly relevant and scored "4". Although exposure through inhalation is expected to be high in the field and may occur within the hive, there are currently no data to determine the level of exposure to in-hive bees by volatile compounds.

- Tables L3 and L4: exposures to dusts from systemic and non-systemic insecticides were considered similar to those from sprayed and non-sprayed from systemic and non-systemic insecticides and, therefore, the same scores were given. The only difference was reported in Table 3.9 for exposure by inhalation where dusts particles were considered too large to be inhaled by bees and therefore exposure by dust vapour in- and out-field was not considered relevant.

- Tables L5 and L6: exposure to soil fumigant was considered very highly relevant for bees only.

In addition to the explanations provided above, some more specific comments were also made directly on the tables for particular categories and cases (see footnotes).

Table L1: Oral exposures from sprayed and non-sprayed (systemic and non-systemic) insecticides

	Foragers	Wax-producing bees	Nurse bees and brood-attending bees	Winter bees[a]	Mating drones	Mating queens	Swarms	Larvae
Oral exposure								
Nectar from plant	4							
Nectar in-hive	4	2[1]	3		3	3	1	1
Honey from nectar	2–4	1	1	4	1	3	3	1
Residues in pollen and bee bread		1[c]	3	1				4
Honey dew	4							
Honey dew in-hive	4	2	3	1	3	3	1	1
Honey from honey dew	2	1	1	4	1	3	3	1
Water (droplets on leaves)[d]	2	1	1	1	1	1		1
Water (leave axils)[d]	1–4?	1	1	1	1	1		1
Water (puddles in field)[d]	1–4?	1	1	1	1	1		1
Water (surface water)[d]	1–4?	1	1	1	1	1		1

As a rule of thumb, in order to produce 1 kg of wax, 7 kg honey is needed.

(a): Winter bees: this category of bees appears in autumn and will spend the winter in the colony. Therefore, their lifespan is not limited to the 3 months of winter, but is longer. Their activity is crucial for the survival of the colony, both in preparing the wintering in autumn and in the recovery of the activity after wintering. In particular, they have to rear the first brood of the new season, which is important for the future development of the colony. The duration of the wintering period is very different among the different European countries, especially between north and south Europe.

(d): This category represents both the water in the field (which can contaminate the foragers) and the water which is brought in the colony by the foragers for two uses: (i) for cooling the colony: the water is deposited in the combs, in particular in the brood comb. After evaporation, the surface of the combs may be contaminated and all categories of bees can be exposed by contact; (ii) for the dilution of some of the honey: the water is distributed to the workers in relation to their needs.

Table L2: Exposures by contact and inhalation from sprayed and non-sprayed systemic and non-systemic insecticides

	Foragers	Wax-producing bees	Nurse bees and brood-attending bees	Winter bees[a]	Mating drones	Mating queens	Swarms	Larvae
Contact/dermal exposure								
Propolis	3	1	1	1	1	1		1–4?

Wax contaminated via pollen and nectar[e]	1	1–3?	1	1	1	1		4
Exposure to soil	1							
Foliar residues (contact)	3							
Interception of droplets (direct overspray)	4				4	4[f]	4	
Residues on pollen	3		3	2				1
Exposure by inhalation[g]								
Vapour in/out the field	4				4	4	4	
Vapour within the hive	1	1–4?	1–4?	1–4?[i]	1	1		1–4?

(a): Winter bees: this category of bees appears in autumn and will spend the winter in the colony. Therefore, their lifespan is not limited to the 3 months of winter, but is longer. Their activity is crucial for the survival of the colony, both in preparing the wintering in autumn and in the recovery of the activity after wintering. In particular, they have to rear the first brood of the new season, which is important for the future development of the colony. The duration of the wintering period is very different among the different European countries, especially between north and south Europe.

(e): In order to renew the old wax combs of their hives, beekeepers buy "new" wax comb foundations. These foundations are built from the old combs which are likely to contain pesticides. Therefore, the "new" wax comb foundations can be contaminated by pesticides and expose all categories of bees in contact.

(f): During mating and orientation flights.

(g): Inhalation includes together volatile substance, micro- and nanoparticles (see the work of Poppy et al.: http://www.sciencedaily.com/releases/2011/10/111007073153.htm?utm_source=feedburner&utm_medium=feed&utm_campaign=Feed:%20sciencedaily%20%28ScienceDaily:%20Latest%20Science%20News%29) and aerosols. Volatilisation of compounds depends of their vapour pressure and of environmental temperature.

(i): The duration of the wintering period is very different among the different European countries, especially between north and south Europe. Therefore, we cannot exclude (especially in south Europe) that winter bees do not bring water inside the colony at the beginning or at the end of the wintering period.

Table L3: Oral exposures from dusts of systemic and non-systemic insecticides

	Foragers	Wax-producing bees	Nurse bees and brood-attending bees	Winter beesa	Mating drones	Mating queens	Swarms	Larvae
Oral exposure								
Nectar from plant	4							
Nectar in-hive	4	2[1]	3		3	3	1	1
Honey from nectar	2–4	1	1	4	1	3	3	1
Residues in pollen and bee bread		1[c]	3	1				4
Honey dew	4							
Honey dew in-hive	4	2	3	1	3	3	1	1
Honey from honey dew	2	1	1	4	1	3	3	1
Water (droplets on leaves)[d]	2	1	1	1	1	1		1
Water (leave axils)[d]	1–4?	1	1	1	1	1		1
Water (puddles in field)[d]	1–4?	1	1	1	1	1		1
Water (surface water)[d]	1–4?	1	1	1	1	1		1

(a): Winter bees: this category of bees appears in autumn and will spend the winter in the colony. Therefore, their lifespan is not limited to the 3 months of winter, but is longer. Their activity is crucial for the survival of the colony, both in preparing the wintering in autumn and in the recovery of the activity after wintering. In particular, they have to rear the first brood of the new season, which is important for the future development of the colony. The duration of the wintering period is very different among the different European countries, especially between north and south Europe.

(c): "+" for pollen in the "wax-producing bees" because honey bees can eat pollen until the age of 32 days (King (1933) in Winston, 1987). The period 1–32 days includes the age of the wax-producing bees.

(d): This category represents both the water in the field (which can contaminate the foragers) and the water which is brought in the colony by the foragers for two uses: (i) for cooling the colony: the water is deposit in the combs, in particular in the brood comb. After evaporation, the surface of the combs may be contaminated and all categories of bees can be exposed by contact; (ii) for the dilution of the some honey: the water is distributed to the workers in relation to their needs.

Table L4: Exposures by contact with and inhalation from dusts from systemic and non-systemic insecticides

	Foragers	Wax-producing bees	Nurse bees and brood-attending bees	Winter bees[a]	Mating drones	Mating queens	Swarms	Larvae
Contact/dermal exposure								
Propolis	2*	1	1	1	1	1		1–4?
Wax contaminated via pollen and nectar[e]	1	1	1	1	1	1		3
Exposure to soil	1							
Foliar residues (contact)	3							
Interception of droplets (direct overspray)	4				4	4[f]	4	
Residues on pollen[b]	3		1–3?	1–3?				
Exposure by inhalation[g]								
Dust vapour in/out-field	NR	NR	NR	NR	NR	NR	NR	NR

(*) only if trees or shrubs providing propolis are treated.
NR, not relevant.
(a): Winter bees: this category of bees appears in autumn and will spend the winter in the colony. Therefore, their lifespan is not limited to the 3 months of winter, but is longer. Their activity is crucial for the survival of the colony, both in preparing the wintering in autumn and in the recovery of the activity after wintering. In particular, they have to rear the first brood of the new season which is important for the future development of the colony. The duration of the wintering period is very different among the different European countries, especially between north and south Europe.
(b): Normally the case of spray of flowering plant should not appear in case of "good practices" because it is not allowed to conduct an insecticide treatment during flowering. However, in case of drifting of the insecticide in the margin of the treated field, bees can be exposed when they forage on the weeds.
(e): In order to renew the old wax combs of their hives, beekeepers buy "new" wax comb foundations. These foundations are built from the old combs, which are likely to contain pesticides. Therefore, the "new" wax comb foundations can be contaminated by pesticides and expose all categories of bees in contact.
(f): During mating and orientation flights.
(g): Inhalation includes together volatile substance, micro- and nanoparticles (see the work of Poppy: http://www.sciencedaily.com/releases/2011/10/111007073153.htm?utm_source=feedburner&utm_medium=feed&utm_campaign=Feed:%20sciencedaily%20%28ScienceDaily:%20Latest%20Science%20News%29) and aerosols. Volatilisation of compounds depends of their vapour pressure and of environmental temperature.
(h): In field there are two possibilities of inhalation of systemics by the honey bees: (i) if the compounds are used in soil or seed treatment, the possibility of volatilisation is very low due to their low vapour pressure; and (ii) if the compounds are sprayed, there is a possibility of inhalation by aerosol.

Table L5: Oral exposures from soil fumigant

	Foragers	Wax-producing bees	Nurse bees and brood-attending bees	Winter bees	Mating drones	Mating queens	Swarms	Larvae
Oral exposure								
Water (puddles in field)	1–4?							
Water (surface water)	1–4?							

Table L6: Exposures by contact and inhalation from soil fumigant

	Foragers	Wax-producing bees	Nurse bees and brood-attending bees	Winter bees	Mating drones	Mating queens	Swarms	Larvae
Exposure by inhalation[g]								
Vapour in/out field	4				4	4	4	

(a): Winter bees: this category of bees appears in autumn and will spend the winter in the colony. Therefore, their lifespan is not limited to the 3 months of winter, but is longer. Their activity is crucial for the survival of the colony, both in preparing the wintering in autumn and in the recovery of the activity after wintering. In particular, they have to rear the first brood of the new season, which is important for the future development of the colony. The duration of the wintering period is very different among the different European countries, especially between north and south Europe.

(e): In order to renew the old wax combs of their hives, beekeepers buy "new" wax comb foundations. These foundations are built from the old combs, which are likely to contain pesticides. Therefore, the "new" wax comb foundations can be contaminated by pesticides and expose all categories of bees in contact.

M. ASSESSMENT OF BUMBLE BEES' EXPOSURE

Table M1: Exposure by oral, contact and inhalation from pesticides in social non-*Apis* bees

Exposure	Source of exposure	Workers	Drones	Queens	Larvae
Oral	Nectar (from plant)	4	2	4	
	Nectar in hive	2	2	2	
	Nectar/honey	2	2	2	2
	Pollen/bee bread	1	1	1	4
	Honey dew[a]	4	2	4	
	Honey dew in hive	2	2	2	
	Honey dew/honey	2	2	2	2
	Water (droplets on leaves)[b]	2		1	
	Water (leave axils)	1		1	
	Water (puddles in field)	1		1	
	Water (surface water)	1		1	
Contact/ dermal	Nesting material wax (contaminated via pollen and nectar)[c]	1	1	1	3
	Exposure to soil[d]	4	4	4	4
	Foliar residues (contact)	3	1	3	
	Interception of droplets (direct overspray)	4	4	4	
	Residues on pollen	3		3	3

Inhalation	Vapour in field	4	4	4	
	Vapour in the soil[d]	4	4	4	4
	Vapour in hive from imported water[e]	1	1	1	1

a. In Thompson (2001): In the UK, no records of bumble bees collecting honey dew from aphids on cereals crops could be found. However, *Bombus lucorum* and other species have been reported to collect honey dew, usually on trees, in Russia, Finland, USA and the UK (see references in Thompson, 2001). For other references in *Bombus* see Konrad et al. (2009b).
b. Need to be confirmed.
c. Only the social group, *Apis*, *Bombus* and Meliponinae (stingless bees), construct their nesting cells, entirely or largely, of materials (wax) secreted from specialised body gland. This wax is secreted by plate-like glands on the sterna (*Apis*) or terga (*Bombus*, *Meliponines*), and is moulded and applied with the mandibles.
d. Several species nest or overwinter in the ground. Exposure is relevant for soil nesting species at the edges of treated fields if the application is during the hibernation period of the queen or during colony development.
e. We need to confirm if other social bees use collect water for nest thermoregulation (e.g. *Bombus*). For example, stingless bees do not use water for cooling their nest (Roubik, 2006).

N. ASSESSMENT OF SOLITARY BEES' EXPOSURE

Table N1: Exposure by oral, contact and inhalation from pesticides in solitary bees

	Adult female	Adult male	Larvae
Oral exposure			
Nectar (from plant)	4	2	
Nectar in hive*[a]			2
Nectar/honey*[a]			2
Pollen/bee bread[a]	1[b]		4
Honey dew [c]	4	1	
Honey dew in hive**[a]			2
Honey dew/honey**[a]			2
Water (droplets on leaves)[d]	2		
Water (leave axils)[e]	1		
Water (puddles in field)[e]	1		
Water (surface water)[e]	1		
Contact/dermal			
Nesting material propolis/resins[f]	4		4
Exposure to soil[f,g]	4	1[h]	4
Foliar residues (contact)[f]	4	1[h]	4
Interception of droplets (direct overspray)	4	4	
Residues on pollen[i]	3		3
Inhalation			
Vapour in/out field	4	4	4
Vapour in the soil	4		4

(*), (**): considering as same category;

a. Food stored by bees ranges from liquid honey to nearly dry, friable masses of pollen. Mass provisioned food (except for the royal jelly to queen honey bee larvae) is always a mixture of pollen and nectar, but it may include a glandular additive.
b. Emerged females ingest pollen from flowers to complete ovary maturation during the pre-nesting period (Kurihara et al., 1981; Richards, 1994).
c. Konrad et al. (2009b) showed that honey dew collection is possible in *Osmia* but it depends on honey dew type and the presence of floral nectar.
d. Need to be confirmed if solitary bees collect guttation drops or dew.
e. Water is particularly necessary in species that nest in the soil. *Antophora* transport quantities of water which are applied to the hard dry surfaces in order to moisten and soften it enough to be able to proceed with the excavation of the tunnel and construction of the turret. Many solitary bees cemented the particles of soil or wood together with water.
f. Many solitary bees used plant products (leave, wood, plant hairs, and resin) as nesting material.
g. Many bees nest in the soil (*Antophora*, *Nomia*) or collect mud as nesting material (*Osmia* spp.).
h. In most non-*Apis* bees, mating occurs on a substrate (soil, flower, leaf, etc.).

i. Bees show different adaptations for pollen transport. *Hylaeus* and other members of the Hylaeinae are the only bees that transport an admixture of pollen and nectar from the flowers to the nest in their crops or honey stomachs. All other bees collect and transport pollen externally. The two main areas of pollen transport on the bodies of bees are the abdominal venter and the hindlegs.

O. FACTORS AFFECTING THE DOSE–LETHALITY RELATIONSHIP

There are several parameters and conditions that may provoke variation of the relationship dose–effect and therefore of LD_{50} values. To reduce variation in estimations of LD_{50}, the following variables, among others, may need to be controlled:

1. the age of the individuals tested (foragers have been shown to be more sensitive to toxicity than the young bees that are normally used for toxicity tests);
2. the nutritional and health status of the bees included in the tests (a low variety of nutritional sources and bees originating from sick colonies have shown to increase toxicity);
3. the previous exposure of the individuals tested to pesticides (sensitisation effect of bees due to previous subchronic exposure to certain pesticides);
4. the genetics of the colony (between different colonies or between different races of bees).
5. the experimental conditions may also cause LD_{50} variation (diverse active substances behaving differently at different temperatures, effects that can also be observed at different concentrations of active substances in the syrup provided to bees).
6. the laboratory performing the methodology, its testing conditions and bee management practices can also make the LD_{50} oscillate.

P. STUDIES ON SUB-LETHAL AND/OR CHRONIC EFFECTS OF PESTICIDES ON HONEY BEES

Reference	Molecules tested and species/subspecies	Study, toxicity and age group	Test dose	Test duration	Endpoints	Results
Aliouane et al. (2009)	**1.** Fipronil **2.** Thiamethoxam **3.** Acetamiprid *A. mellifera*	Lab study Oral + contact exposures Adults	**1.** 0.1–0.01 ng/bee **2.** 1–0.1 ng/bee **3.** Oral: 1–0.1 µg/bee	11 days	Behavioural functions	**1.** Mortality at 0.1 ng/bee after 1 week **2.** Induced by contact either a significant decrease in olfactory memory 24 h after learning at 0.1 ng/bee or a significant impairment of learning performance with no effect on memory at 1 ng/bee. Responsiveness to antennal sucrose stimulation was significantly decreased at high sucrose concentrations (1 ng/bee) **3.** Increase in responsiveness to water
Attencia et al. (2005)	**1.** Methyl parathion (MeP) and **2.** Malathion *A. mellifera*	Lab study In-hive exposure with filter paper Adults	**1.** 0.001 %, 0.01 %, 0.05 % and 0.1 % **2.** 0.02 %, 0.05 %, 0.5 % and 1 %	On 1, 7, 14, 21-day-old bees	Alteration of esterase activity in bee body	**1.** Esterase 1 activity reduced at 0.01 % and esterase 2 increased in 14- and 21-day-old bees, Esterase 3 and 4 50 % inhibited in 1-day-old bees but increased in 14-day-old bees; at 0.05 % esterase activity affected to varying degrees
Aupinel et al. (2007)	**1.** Dimethoate **2**. Fenoxycarb *A. mellifera*	Lab study Oral exposure Larvae	**1.** Acute: 0.8, 1.6, 3.2, 6.4, 12.8 µg/larva Chronic: 2.5, 5, 10, 20, 40 mg/kg	**1.** Day 4 for acute and day 1–6 for chronic	Larval mortality at days 7 and 22	**1.** LD_{50} (48 h) = 1.9 µg/larva (day 4) NOAEC (day 7) = 2.5 mg/kg NOAEC (day 22) = 5 mg/kg **2.** No effect at day 4; emergence affected at 6 ng/larva

Beliën et al. (2010)	**1.** Imidacloprid **2.** Fenoxycarb **3.** Indoxacarb *A. mellifera*	Field study Oral exposure with syrup in hive Adults + brood	**1.** 1 ppb **2.** 100 ppm **3.** 300 ppb	Every 2 weeks for 10 weeks; foraging behaviour was measured on individual bees aged 13, 15, 18, 20 and 25 days	Foraging activity (NB: active and dead bees; surface of capped brood, colony weight); foraging behaviour (phototaxis)	First 6 weeks: normal population size and drop during the next 4 weeks, significantly after 6 weeks. Total number of active bees and caped brood cells decreased after 6 weeks. The timing of the positively phototactic behaviour is influenced by fenoxycarb
Beliën et al. (2009)	**1.** Imidacloprid **2.** Fenoxycarb **3.** Indoxacarb *A. mellifera carnica*	Field study Oral exposure with syrup in hive Adults + brood	350 ml sugar water solution with: **1.** 3.55 ng a.i./l **2.** 10.21 mg a.i./l **3.** 1 g a.i./L	Every 2–7 days, after several weeks after treatment	Foraging activity (NB: active and dead bees; surface of capped brood, colony weight) and mortality	Decay of overall colony vitality for several hives a couple of weeks after treatment
Bernadou et al. (2009)	Fipronil *A. mellifera*	Lab study Contact exposure Adults	0.5 ng/bee	3–24 h	Learning	**1.** Decreased acquisition success **2.** Subsequent memory performances lowered **3.** No effect on distribution of responses to the tactile stimuli between sides
Bendahou et al. (1999)	Cymbush (Cypermethrin) *A. mellifera mellifera*	Field study Oral exposure Adults + brood	12.5 µg/L	5 months (July–November)	Mortality in the hive, bee behaviour, brood areas, supersedure, glucosaemia, trehalosaemia, and Na^+,K^+-ATPase activity	Supersedure and brood absorption significantly higher in treated colonies than in controls. Also, significant differences well treated colonies and controls in glucosaemia and trehalosaemia (different trends over time – hypo trends in treated colonies at weeks 3, 8, 12, 16, 18), significant differences in ATPase activity (in treated colonies deficit and inhibition between 20 % and 35 %)
Bortolotti et al. (2003)	Imidacloprid *A. mellifera*	Field + feeder Oral exposure Adults	100 ppb, 500 ppb, 1 000 ppb	+1–2 h, +5, +24 h	Feeding, foraging and homing behaviours	Repellence at feeder for 500–1000 ppb Return to hive after a delay of 24 h at 100 ppb and did not return at 500–1 000 ppb

Colin et al. (2004)	**1.** Imidacloprid 2**.** Fipronil *A. mellifera*	Semi-field + feeder Oral exposure Adults	**1.** 6 ppb **2.** 2 ppb	Eight control colonies during 5 days at different times of the season and three colonies before contamination and during 4 days after	Foraging activity at feeders in tunnels	**1.** Decrease in the proportion of active bees at the feeder **2.** Additional decrease in attendance at the feeder
Cruz et al. (2010)	**1.** Boric acid (BA) **2.** Fipronil Africanised *A. mellifera*	Lab study Oral exposure Larvae	**1.** 1, 2.5, 7.5 mg/g **2.** 0.1, 1 µg/g	**1.** 4 days **2.** 3 days	Larvae mortality, morphological alterations of midgut	Morphological alterations with boric acid greater than with fipronil
Cutler and Scott-Dupree. (2007).	Clothianidin Honey bees	Field study Oral exposure Adults + brood	400 g a.i./100 kg seed	From spring in year *n* to spring in year *n* + 1	Colony weight gain, honey production; adult mortality, brood development, longevity	No significant effect
Dai et al. (2010)	**1.** Bifenthrin **2.** Deltamethrin *A. mellifera ligustica*	Lab + field studies Oral exposure with syrup to queen and in hive Adults + eggs + larvae	**1.** 4, 7.9, 15.5, 30.6, 60.2 mg/L **2.** 20, 36, 64.8, 116.6, 210 mg/L	Mortality after 48 h; fecundity, growth and development on 3 days over 3 years (more detailed timing in the article for all detailed endpoints)	Daily fecundity, growth and development (egg and larva weights, hatch cap emergence rate, the success rate of development, the egg stage, the unsealed brood stage, sealed brood stage, immature stage)	**1 + 2.** Fecundity and growth reduced; immature stage increased **1.** Median lethal effect = 16.7 mg/L **2.** Median lethal effect = 62.8 mg/L
Decourtye et al. (2011)	Fipronil *A. mellifera ligustica*	Semi-field study + feeder Oral exposure Adults	0.06 and 0.3 ng/bee	6–7 h out of the tunnel	Foraging with RFID (time spent in the hive, at the feeder, between the feeder and hive, number of entries and exits from the hive and at the feeder	0.3 ng reduced the number of foraging flights and prolonged the duration of homing flights over 3 days
Decourtye et al. (2009)	Fipronil *A. mellifera* L.	Outdoor flight cage + feeder Oral exposure Adults	1 µg/kg		Orientation capacities	NB: foragers entering are is reduced and rate of finding correct path is reduced from 89 % to 60 % (and 4 % of bees do not find the path within 5 min in control and 34 % in treated)

Decourtye et al. (2005)	Nine pesticides (fipronil F, deltamethrin D, endosulfan E, prochloraz P, i-cyalothrin Cya, cypermethrin Cyp, I-fluvalinate Fl, triazamate T, dimethoate Di) *A. mellifera ligustica*	Lab study Oral exposure Adults	Highest tested dose is LD_{50} 48 h/20 per bee per day	2 to 14–15-day-old bees treated daily during 11 days	Proboscis extension response (PER) on 2- to 15-day-old bees	PER reduced for F, D, E, P No PER impairment for all other pesticides
Decourtye et al. (2004a)	Imidacloprid	Lab study Oral exposure Adults	12 and 0.12 ng/bee	Short- (30 s to 3 min), mid- (15 to 60 min) and long-term (24 h)	PER (OL)	Impairment of OL and increase in cytochrome oxidase (CO) in mushroom bodies. No effect on short-term (0 s–3 min) and long-term (24 h) term OL
Decourtye et al. (2004b)	**1.** Imidacloprid **2.** Deltamethrin *A. mellifera ligustica*	Lab and semi-field studies + feeder Oral (feeding + foraging) and contact (PER) exposures Adults	**1.** 24 µg/kg **2.** 500 µg/kg	Recording visits at scented/unscented sites every 30 s for 5 min; bee counter to measure colony activity at the hive entrance in June–July	Foraging activity, associative learning	Decrease in foraging activity on the food source and activity at the hive entrance Deltamethrin had lethal effect on workers bees As with free-flying bees, no impact of deltamethrin was found on the learning performances of restrained individuals in the PER procedure. Significant effects were found with imidacloprid in both semi-field and laboratory conditions
Decourtye et al. (2003)	**1.** Imidacloprid **2.** 5-OH-imidacloprid *A. mellifera ligustica*	Lab study Oral exposure Adults	**1.** 2–32 ng/bee **2.** 12.5–200 ng/bee	Acute toxicity at 48 h on workers of unknown age (summer bees from August to October; winter bees from December to February). PER on winter and summer bees (July) exposed from day 2 to 14–15	Mortality, PER, foraging	LD_{50} (48 h) is 30.6–153.5 ng/bee LOEC of 1 and 2 on mortality of winter bees were 24 ppb and 120 ppb, respectively. Winter bees surviving chronic treatment with 1 and 2 had reduced learning performances. The LOEC of 1 in summer bees (12 ppb) was lower than in winter bees (48 ppb). Chronic toxicity lower in winter bees (48 ppb) than in summer bees (96 ppb)

El Hassani et al. (2009)	Fipronil Honey bees	Lab study Exposure through injection on thorax Adults	0.1 or 0.5 ng/bee at +20 and +60 min	1 h, 24 h, 48 h	PER (OL) and neurotransmission (GABA/glutamate-gated chloride channels)	OL impaired at 0.1 ng/bee OL not impaired at 0.5 ng/bee
El Hassani (2008a)	**1.** Acetamiprid **2.** Thiamethoxam Honey bees	Lab study Oral + contact exposures Adults	Contact: 1 µl (10 % solvent) Oral: **1.** 0.1, 0.5, 1 µg/bee **2.** 0.1, 0.5, 1 ng/bee	Locomotor activity tested after 1 h of contact or oral application Sucrose sensitivity: 1 h before and after treatment Olfactory learning: treatment 3 h before conditioning and recording 1 h, 24 h, 48 h after	Locomotor activity, sucrose sensitivity, OL	**1.** Oral: antennal stimulation sensitivity increased at 1 µg/bee and long-term OL at 0.1 µg/bee; contact: no effect on OL but increased locomotor activity at 0.1–0.5 µg/bee and PER at 0.1, 0.5 and 1 µg/bee **2.** No effect
El Hassani et al. (2008b)	**1.** Ivermectin **2.** Ivermectin + L-trans-PDC or TACA Honey bees	Lab study Exposure through injection on thorax Adults	**1.** 0.01 ng/bee and 0.05 ng/bee	1 h, 24 h, 48 h	Olfactory memory (OM)	**1.** At low dose (48 h) OM impaired; at higher dose no effect **2.** Low dose of **1** impaired OM (48 h) and the effect was rescued by injection of both L-trans-PDC and TACA. Higher dose of 1 + TACA impaired OM (48 h)
El Hassani et al. (2005)	Fipronil *A. mellifera*	Lab study oral + contact exposures Adults	Oral: 0.1, 0.5 and 1 ng/bee + 0.01 ng/bee for PER Topical: 0.1, 0.5, 1 ng/bee	1 h before and 1 h after treatment	Sucrose sensitivity (SS), locomotor activity (LA), olfactory learning (OL)	Contact: 1 ng/bee decreases SS 1 h after treatment and 0.5 ng/bee impairs OL Oral or contact: no effect on LA

Falco et al. (2010)	Thiamethoxam *A. mellifera*	Lab study Oral exposure Adults	6 µg/mL, 3 µg/mL, 1.5 µg/mL, 0.5 µg/mL, 0.05 µg/mL, 0.005 µg/mL	0-, 7-, 14- and 21-day-old bees in boxes tests with feeder for 24 h	Mortality, acceptance/rejection of food, chromatin of Malpighian tubules (CEC); in 0-, 7-, 14- and 21-day-old bees	*Mortality* *0 days: 0 % at 5 ng, 83 % at 50 ng *7 days: 12.5 % at 5 ng, 54 % at 0.5 µg *14 days: 1 % at 5 ng, 80 % at 0.5 µg *21 days: 5 % at 5 ng, 95 % at 0.5 µg *Food* Rejection at high dose and acceptance at low dose *CEC* (tbd)
Gauthier et al. (2009)	Fipronil *A. mellifera*	Lab study Oral + contact exposures Adults	Acute: 1, 0.5, 0.1 ng/bee chronic: 0.1, 0.01 ng/bee	Foraging bees (acute toxicity) and emerging bees (chronic) for 11 days	Memory, learning, odour sensitivity	DL_{50} = 5 ng/bee Contact: acute: 1 ng/bee decreases sensitivity to sugar solution (low concentration) 0.5 ng/bee affects learning and memory (PER) Chronic exposure at 0.1 ng/bee leads to 100 % mortality, at 0.01 ng/bee (contact) reduction in locomotion and increased water consumption; 0.01 ng/bee (contact or oral) decreases odour discrimination
Guez et al. (2005)	Methyl parathion (MeP) *A. mellifera ligustica*	Flight cage study + feeder Contact exposure Adults	10 and 50 ng/bee	Recording during 1 h after treatment	Frequency of visits of foragers at a feeder	At 10 ng/bee, decrease followed by increase; at 50 ng, increase

Guez et al. (2001)	Imidacloprid *A. mellifera*	Lab study Contact exposure Adults	0.1, 1, 10 ng/bee	15 min, 1 h, 4 h after application	PER	In normal conditions PER requires more days in older (8–10 days) than younger (4–7 days) bees In 7-day-old bees: imidacloprid increases the number of trials required In 8-day-old bees imidacloprid decreases the number of trials required
Heylen et al. (2010)	**1.** Captan **2.** Imidacloprid **3.** Indoxacarb **4.** Fenoxycarb *A. mellifera carnica*	Lab study Oral exposure Adults	$LD_{50}/_{10}$: **1**. 120 ppm **2.** 1 ppb **3.** 300 ppb **4.** 100 ppm	Experiment during two consecutive summers; treatment over 24 h **1–3.**1 day + 7 days after treatment **4.** 1, 2, 3, 4, 5, 7 days after treatment	Size and morphology of hypopharyngeal gland (HPG) in 8- and 14-days-old bees	**1–3.** No effect **4.** HPG decreased in size, a granular texture and unorganized cytoplasm more quickly. At 7 days, HPG typical of a forager
Kirchner (1999)	Imidacloprid	Field study	20–100 µg/kg		Foraging and waggle dance	Foraging and dances affected at 20 µg/kg
Lambin et al. (2001)	Imidacloprid *A. mellifera*	Lab study Contact exposure Adults	1 µL at 1.25, 2.5, 5, 10, 20 ng/bee	At 0, 15, 30 and 60 min after treatment	Non-associative learning abilities (PER) and motor activity	5 ng/bee and above has an effect on the gustatory function; 2.5 ng/bee and above impairs motor activity in open field (these effects are amplified over time). Motor activity is enhanced and PER habituation is facilitated at doses below 2.5 ng/bee
MacKenzie and Winston (1989)	**1.** Diazinon **2.** Carbaryl **3.** Resmethrin *A. mellifera*	Lab and field studies Contact exposure Adults	1 µL LD_{25}, LD_{10}, LD_5	Bees at 0 and 14 days old; three colonies	Longevity, foraging age	**1–3.** Descriptions of LD_5, LD_{10} and LD_{25} For 0-day-old bees carbaryl is the most toxic at sublethal doses and impacts both longevity and foraging age; carbaryl is the least toxic at acute doses; emerging bees are more sensitive to pesticides than older bees (at both acute and sublethal doses)

Medrzycki et al. (2003)	Imidacloprid *A. mellifera*	Lab study Oral exposure Adults	100, 500 ppb at single dose and *ad libitum*	Five recordings (0–30 min; 30–60 min; 1–2 h; 6.5–7 h; 23–23.5 h) during 24 h	Mobility	Mobility is reduced; effects start at 30–60 min after ingestion and disappear after a few hours; loss of communicative capacity
Schmuck (2004)	**1.** Metabolites of imidacloprid (urea NTN and 6-CAN) *A. mellifera*	Lab study Oral exposure Adults of two age cohorts (emerging versus foragers)	0.1, 1 and 10 µg/L	24 h, 48 h, 10 days	Mortality, knocked down, staggering, responsiveness	No significant effects
Schneider et al. (2012)	**1.** Imidaclorpid **2 .** Clothianidin *A. mellifera carnica*	Field study + feeder Oral exposure (bees treated in lab) Adults	**1.** 0.15, 1.5, 3, 6 ng/bee **2.** 0.05, 0.5, 1, 2 ng/bee	Immediately after treatment for 3 h and between 24 and 48 h	Foraging behaviour: number of trips from hive to feeder, duration of trips, time interval between trips	**1.** At 3 ng 95 % of bees return to the hive compared with 25 % at 6 ng (among the 5 % at 3 ng and 75 % at 6 ng trembling, reduced mobility, cleaning). Visit frequency to feeder reduced at 1.5 and 3 ng/bee. **2.** At 1 ng 73.8 % returned to the hive compared with 20.6 % at 2 ng. At 0.5–2 ng reduced visit frequency; reduced number of bees returning to the feeder (67.8 % at 1 ng and 11.8 % at 2 ng). Effects did not persist on day2
Smodis Skerl and Gregorc (2010)	**1.** Imidacloprid **2.** Coumaphos *A. mellifera carnica*	Lab study Oral exposure Adults	**1.** 0.5 ppb	**1.** 24 h, 48–7 h **2.** 24 h, 48–72 h	Hypopharyngeal glands (HPG) acinus diameter in 1–6, 7–12, 13–18, and 19- to 32-day-old bees	**1.** HPG size variations **2.** Heat shock protein 70 (Hsp70) localisation in nuclei and cytoplasm and Hsp90 activity in cell cytoplasm → coumaphos triggers increase level of programmed cell death and imidacloprid induces extended necrosis (50 % cell death after 48 h and 100 % after 72 h)

Stadler et al. (2003)	Imidacloprid *A. mellifera ligustica*	Field study Oral exposure Adults + brood	Seeds treated with 0.24 mg a.i./seed	Long-term (226 days: 10 days in the field and observations on the remaining 216 days)	Population development and honey production (bee hive weight, nectar, pollen, brood, honey production, foraging activity, pollen entrance and mortality)	No significant difference for their development regarding pollen entrance and pollen in the hives, nectar and mortality; Treated hives were more productive (higher weight, honey production, foraging activity, brood); high proportion of sunflower pollen in both treated and controls
Stanley et al. (2010)	Diafenthurion *A. cerana indica*	Lab and field studies Contact exposure Adults Semi-field Oral exposure Adults	Lab: 0.2, 0.5, 1, 4, 10, 20, 40, 80, 140, 200, 250 and 300 µg/bee Semi-field: 3 and 30 µg/ml Field: 750 L/ha	Lab: In 3-day-old bees, mortality at 3, 6, 12, 24, 48 h after treatment; Semi-field: Observations at the hive over 1 h; observations at the feeder 24 h after treatment over 2 h and then over 12 h in the lab to record mortality and observations on another lot of bees after 3 days Field: bee count over 30 min	Mortality, foraging and homing behaviour	Semi-field: control bees left the cage immediately and bees at 30 µg/ml took 14 min. Contaminated bees took longer to return to the hive than control bees (this was exacerbated over time and fewer bees returned at higher concentrations); median lethal time was 85.8 h at 30 µg/ml. Field: no impact
Suchail et al. (2001)	**1.** Imidacloprid **2.** 5-Hydroxymidacloprid; 4,5-dihydroxyimidacloprid; desnitroimidacloprid, 6-chloronicotinic acid; **3.** Olefin; urea derivative *A. mellifera*	Lab study Oral acute and chronic exposures Adults	0.1, 1, 10 µg/L for 10 days and cumulative dose 0.01, 0.1, 1 ng/bee after 8 days	10 days 8 days	Mortality; hyper-responsiveness, hyperactivity, trembling	50 % mortality after 8 days **1.** LD_{50} = 60 ng/bee after 48 h LD_{50} = 40 ng/ab after 72 and 96 h **2.** LD_{50} 5-hydroxymidacloprid > LD_{50} imidacloprid **3.** LD_{50} olefin < LD_{50} imidacloprid
Thompson et al. (2007)	**1.** Fenoxycarb **2.** Diflubenzuron	Field + feeder study Oral exposure Adults + brood	**1.** 750 mg/L **2.** 288 mg/L	1 week exposure; observations up to 14 days and 2.5 months after treatment	Brood development, mortality, longevity	Brood mortality has little effect on population size; precocious foraging in affected bees and shortening of the time a nurse can produce food and rear brood

Wu et al. (2011)	39 pesticide residues in combs (7 fungicides, 2 herbicides, 23 insecticides and 7 metabolites	Field study Contact exposure Adults + brood	Residues detection from 1 to 8079 ng/g of comb (depending on the active ingredient) in treatment combs	Egg eclosion at day 4, larval mortality at day 8, pupation at days 12 and 19, adult emergence from day 20, adult longevity daily; multiple brood cycles	Brood mortality, emergence, development, longevity	Delayed development for bees aged 4 and 8 days; adult longevity reduced by 4 days; higher brood mortality and delayed adult emergence after multiple brood cycles; increased adult survival after multiple brood cycles
Yang et al. (2008)	Imidacloprid Sp?	Field + feeder Oral exposure (lab treatment) Adults	50, 100, 200, 400, 600, 800 µg/L	Continuous recording of feeding intervals for 1 h before treatment and for 1.5 h after treatment	Time interval between foraging visits	In controls, time interval is < 300 s; in treated bees time interval is > 300 s at all concentrations (after 20 min at 50 µg/L and after 10 min at 100 µg/L); calculation of bees' consumption show possible behavioural disruption at 1.82–4.3 ng/bee

Q. STUDIES ON SUBLETHAL AND/OR CHRONIC EFFECTS OF PESTICIDES ON SOCIAL NON-*APIS* (BUMBLE BEES) AND SOLITARY BEES

References	Molecules tested Species/subspecies	Study Toxicity Age group	Test dose	Test duration	Endpoints	Results	Notes
Abbott et al (2008)	**1.** Imidacloprid **2.** Clothianidin *Megachile rotundata* *Osmia lignaria*	Oral exposure Larvae	Low (3 or 6 ppb), intermediate (30 ppb), or high (300 ppb)	Total development period	Mortality rate, Development duration, adult weight	No lethal effects of imidacloprid or clothianidin on *O. lignaria* and *M. rotundata* Minor sublethal effects on larval development of *O. lignaria*, with greater developmental time at the intermediate (30 ppb) and high doses (300 ppb) of imidacloprid	
Babendreier et al. (2008)	Insecticidal proteins: **1.** Kunitz soybean trypsin inhibitor (SBTI) **2.** *Galanthus nivalis* agglutinin (GNA) **3.** Bt toxin (Cry1Ab) *Bombus terrestris*	Oral (chronic) exposure Adults	0.01 % Cry1Ab, 0.1 % and 0.01 % SBTI, and 0.1 % and 0.01 % GNA	Maximum: 80 days	Survival in bumble bee microcolonies*	While the Cry1Ab did not affect microcolony performance, the consumption of SBTI and especially GNA affected survival of *B. terrestris* workers and drones and caused a significant reduction in the number of offspring	*The use of microcolonies appears to be well suited to measure lethal and sublethal effects of insecticidal proteins expressed in transgenic plants or systemic insecticides on bumble bees

Besard et al. (2010)	23 acaricide products (active substances listed): **1.** Dienochlor **2.** Bifenthrin **3.** Abamectin **4.** Milbemectin **5.** Clofentazine **6.** Hexythiazox **7.** Etoxazole **8.** Azocyclotin **9.** Fenbutatin oxide **10.** Clorfenapyr **11.** Flucycloxuron **12.** Amitraz **13.** Acequinocyl **14.** Fenazaquin **15.** Fenpyroximate **16.** Pyridaben **17.** Tebufenpyrad **18.** Tebufenpyrad **19.** Tebufenpyrad **20.** Spirodiclofen **21.** Spiromesifen **22.** Bifenazate **23.** Bromopropylate *Bombus terrestris*	Topical and oral application via pollen and sugar solution	Maximum field recommended concentration (mg a.i./L): **1.** 500 **2.** 30 **3.** 18 **4.** 10 **5.** 150 **6.** 3 **7.** 55 **8.** 750 **9.** 275 **10.** 240 **11**. 125 **12.** 400 **13.** 150 **14.** 200 **15.** 50 **16.** 75 **17.** 100 **18.** 200 **19.** 10 **20.** 96 **21.** 0.8 **22.** 96 **23.** 500 In the case of the four most toxic compounds (bifenthrin, abamectin, etoxazole and bifenazate), the LC_{50} was calculated using oral exposure via treated sugar water	11 weeks	Lethal and effects on drones production	Several chemistries caused high levels of acute toxicity and detrimental effect on drone production in bumble bee workers at MFRC. LC_{50} values: abamectin (1.17 mg/L), bifenazate (9.6 mg/L), bifenthrin (0.36 mg/L) and etoxazole (4.4 mg/L)	

Besard et al. (2011)	**1.** Spinetoram **2.** Spinosad *Bombus terrestris*	Direct contact with wet and dry residues and oral exposure Adults	From the MFRC* to four different dilutions (1/10, 1/100, 1/1 000, 1/10 000 of the MFRC)	From 72 h (acute) to 11 weeks (chronic)	Lethal and sublethal effects on aspects of bumble bee reproduction and foraging behaviour	**1.** NOEC = 1/100 of the MFRC; **2.** NOEC = 1/1 000 of the MFRC; No effects on nest reproduction at the corresponding NOEC values for spinetoram and spinosad.	
Franklin et al. (2004)	Clothianidin *Bombus impatiens*	Oral via pollen (chronic exposure) Colony	6 and 36 ppb	> 80 days	Pollen consumption, progeny weight, number of males, queens and workers, foraging behaviour	No effects were observed. The foraging ability of the workers bees tested on artificial flowers also did not differ among treatments	
Gradish et al. (2012)	**1.** Flubendiamide **2.** Deltamethrin **3.** Spinetoram *Megachile rotundata*	Oral exposure Larvae	0.1, 0.2, 0.05 mg a.i./kg of pollen	Total development	Survival during development and percentage of emergence	Larvae treated with deltamethrin and spinetoram in the laboratory either died before spinning a cocoon or, in the case of spinetoram, occasionally pupated without spinning a cocoon. Flubendiamide had no effect on larval survivorship, or time to complete cocoon spinning	

Hodgson et al. (2011)	Novaluron *Megachile rotundata*	Oral exposure Adults and larvae	From one-half to 10 times the field rate (745 mL/ha)	Development period	Immature mortality rate	In laboratory conditions, immature mortality rate at all nuvaluron provision dosing treatments was significantly higher than among the water or blank controls. In field cages, at least 84 % of progeny died	
Konrad et al. (2008)	The insecticidal proteins*: **1**. Recombinant orizacystatin-1 (rOC-1) **2.** *Galanthus nivalis* agglutinin (GNA) **3.** Bt toxin (Cry1Ab) *Osmia bicornis* (=*O. rufa*)	Oral exposure Larvae	rOC-1 (0.1 % of fresh pollen provision w:w), GNA (0.01 % and 0.1 %), and Cry1Ab (0.01 %)*	Total development and adult longevity	Larval development, mortality, body weight and adult longevity	High doses of rOC-1 and Cry1Ab as well as a low dose of GNA failed to cause any significant effects. However, a high dose of GNA (0.1 %) resulted in significantly increased development time and reduced efficiency in conversion of pollen food into larval body weight	*The insecticidal proteins were tested in purified form by adding them to the pollen of control oilseed rape plants (50 µg/g provision)
Konrad et al. (2009a)	The insecticidal proteins*: **1.** Recombinant orizacystatin-1 (rOC-1) **2.** Kunitz soybean trypsin inhibitor (SBTI) **3.** *Galanthus nivalis* agglutinin (GNA) **4.** Bt toxin (Cry1Ab) *Osmia bicornis* (=*O. rufa*)	Oral exposure Adults	0.0 %1 and 0.1 %, w:v, Cry1Ab only at 0.01 %	Longevity of adult chronically exposed to test solutions	Bee mortality and activity, effects of proteinase inhibitors on *Osmia bicornis* digestive proteolytic activity	Longevity was significantly reduced by SBTI and GNA at both concentrations and by rOC-1 at 0.1 %. A relatively complex profile of at least four types of soluble proteolytic enzymes was identified	

Johansen et al. (1984)	**1.** Aldicarb **2.** Aldicarb sulfoxide **3.** Aldicarb sulfone *Megachile rotundata* *Nomia melanderi*	Oral exposure Adults	Several concentrations (chronic feeling test)	Up to 21 days	LC_{50} at 21 days	*Nomia melanderi* **1.** LC_{50}: 2.0 ppm **2.** LC_{50}: 2.0 ppm **3.** LC_{50}: 8.4 ppm *Megachile rotundata* (♀) **1.** LC_{50}: 3.6 ppm **2.** LC_{50}: 2.1 ppm **3.** LC_{50}: 5.6 ppm	
Ladurner et al. (2005)	**1.** Captan **2.** Benomyl **3.** Propiconazole **4.** Iprodione **5.** Neem oil *Osmia lignaria* *Apis mellifera*	Oral* and contact exposure Adults	**1.** 122.5 µg/bee **2.** 125 µg/bees **3.** 65 (oral), 104 (contact) µg/bee **4.** 125 µg/bee **5.** 196.4 µg/bee	24 h, 48 h, 72 h, up to 7 days	Delayed toxicity (any negative side-effects occurring after a period of > 72 h from the application of a single dose of test substance)	Exposures to benomyl and iprodione did not affect survival of any of the two species. Contact exposure to neem oil affected survival of *A. mellifera*. Orally administered propiconazole showed delayed and acute toxicity to both species. Captan severely limited survival of *O. lignaria*	*Bees were individually fed 10 µL of test solution using the flower method (Ladurner et al., 2003)
Ladurner et al. (2008)	**1.** Iprodione **2.** Propiconazole **3.** Benomyl **4.** Captan **5.** The surfactant Dyne-Amic alone **6.** Dyne-Amic mixed with iprodione **7.** Tank mixture IDB (iprodione + Dyne-Amic + foliar fertiliser Bayfolan Plus) *Osmia lignaria*	All ways of exposure (cage study) Adults	All treatments were applied at the highest recommended field rate for stone fruits with hand-held sprayers, at a rate of 1021 litres of water per ha	Day 0 (day of treatment), and on days 1, 2, and 4 after application	The time spent inside the nest depositing pollen-nectar loads, the foraging time, the cell production rate, and the survival were recorded for each female *O. lignaria*	A high proportion of females in the IDB cage were inactive for a few hours before resuming normal foraging and nesting activity. No lethal or behavioural effects were found for any of the other compounds or mixtures tested	

Mommaerts et al. (2006)	Three juvenile hormones analogues: **1.** Pyriproxyfen **2.** Fenoxycarb **3.** Kinoprene Two ecdysone agonists: **4.** Tebufenozide **5.** Methoxyfenozide *Bombus terrestris*	Dermal, oral (via sugar solution or pollen) exposure/adults	MFRC (mg/L): 1. 25, 2. 100, 3. 650, 4. 240, 5. 96	11 weeks	Lethal and effect on male production, ovary development and progeny mortality	These risk hazard tests showed that the tested IGRs caused no acute toxicity on the workers, and any compound had an adverse effect on reproduction (production of males). However, in the nests where the workers were exposed to pyriproxyfen and kinoprene* higher numbers of dead larvae were scored	Kinoprene at lower concentrations (0.0650 mg a.i/l) had a stimulatory effect on brood production. It was remarkable that ovaries of such treated dominant workers were longer and contained more eggs than in the controls.
Mommaerts et al. (2009)	Seven microbiological control agents (MCAs): **1.** *Ampelomyces quisqualis* 2. *Hypocrea parapilulifera* + *Trichoderma atroviride* **3.** *Gliocladium catenulatum* J1446 **4.** *Bacillus subtilis* QST713 **5.** *Trichoderma harzianum* T22 **6.** *Beauveria bassiana* GHA **7.** *Cydia pomonella* granulovirus *Bombus terrestris*	Dermal contact and orally via either treated sugar water or pollen Adults	MFRC	Up to 11 weeks	Lethal and sublethal effects on drone production, foraging behaviour	The tested MCAs were found to be safe for workers of *B. terrestris*, with the exception of *Beauveria bassiana* and *Bacillus subtilis*. These MCAs showed also sublethal effects on drone production (*B. bassiana* and *B. subtilis* at 1/2, 1/5 and 1/10 of MFRC) and on foraging behaviour (*B. bassiana* at its MFRC)	

Mommaerts et al. (2010a)	*Bacillus thuringiensis* strains: kurstaki and aizawai *Bombus terrestris*	Dermal contact and oral feeding via treated pollen and treated sugar water (chronic exposure) Adults	0.1 % of each compound, representing the MFRC	11 weeks	Lethal and sub-lethal hazards on colony reproduction and foraging behaviour of workers	The Bt strains are safe to *B. terrestris* bumble bees, although in some cases there were detrimental effects that depended on strain and route of exposure. In particular, aizawai killed all workers at a concentration of 0.1 % when applied in the feeding sugar water and reduced reproduction when applied in pollen	
Mommaerts et al. (2010b)	**1.** Imidacloprid **2.** Thiametoxam **3.** Thiacloprid *Bombus terrestris*	Oral exposure Adults	From the MFRC to several dilutions: **1.** From 200 ppm to 10 ppb **2**. From 100 ppm to 10 ppb **3.** From 120 ppm to 12 ppb	Up to 11 weeks (chronic exposure)	Mortality, drone production and foraging behaviour	Chronic toxicity assay without and including foraging (only for imidacloprid-lower value in the range). **1.** LC_{50} = 20–59 ppb (NOEC for survival = 10 ppb); EC_{50} = 3.7–37 ppb (NOEC for reproduction = < 2.5–20 ppb) **2.** LC_{50} = 0.12 ppm; EC_{50} = 35 ppb **3.** LC_{50} = 18 ppm; EC_{50} = 12 ppm)	This study reports the development of a new bioassay to assess the impact of sublethal concentrations on the bumble bee foraging behaviour under laboratory conditions

Mommaerts et al. (2011)	Perfluorooctane sulfonic acid (PFOS) *Bombus terrestris*	Oral (chronic) exposure Adults	From 1 µg/L to 10 mg/L	11 weeks	LC_{50}, ovary size, survival and reproduction capacity, mitochondrial electron transport activity, lipid, protein and glucose amounts, EcR interaction of PFOS with ecdysteroid-responsive insect S2 cells	A chronic toxicity assay demonstrated high bumble bee worker mortality (up to 100 %) with an LC_{50} of 1.01 mg/l. In addition, PFOS posed strong detrimental reproductive effects, and these combined with a dramatic reduction in ovarian size. Effects on mitochondrial electron transport activity and energy content were observed	
Morandin and Winston (2003)	**1.** CryIAc **2.** Chitinase **3.** Imidacloprid *Bombus impatiens* (experiment 1) *Bombus occidentalis* (experiment 2)	Oral via pollen exposure/colony	Experiment 1– realistic residue level in pollen: **1.** 11 ng/g pollen **2.** 0.6 µg/g pollen **3.** 7 ng/g pollen; Experiment 2: **1.** 11 ng/g pollen; **3.** 7 and 30 ng/g pollen	11 weeks	Pollen consumption, bumble bee worker weights, colony size, amount of brood, queens and drones produced and foraging ability on artificial flowers (only in experiment 2)	No effects in bumble bee exposed to CryIAc, chitinase and imidacloprid at concentrations similar to the highest residue levels found in pollen. However, when *B. impatiens* colonies were exposed to imidacloprid at 30 ppb, access times and foraging rates of individual bees were slower than those of bees exposed to 7 ppb imidacloprid or controls*	*Use of an artificial flower foraging array proved to be a sensitive method for detecting sublethal response of bees to pesticides

Morandin et al. (2005)	Spinosad *Bombus impatiens*	Oral exposure Larvae	0.2, 0.8 and 8 mg/kg in pollen	10 weeks	Adult mortality, brood development, weights of emerging bees and foraging efficiency of adults	Spinosad at a level of 8 mg/kg in pollen was clearly detrimental to bumble bee colony health, resulting in colony death 2–4 weeks after initial exposure. At more realistic concentrations there were potentially important sublethal effects. Adult worker bees exposed to spinosad during larval development at 0.8 mg/kg were slower foragers on artificial complex flower arrays than bees from low- or no-spinosad-treated colonies	
Peach et al. (1995)	Carbaryl *Megachile rotundata*	Oral exposure Adults*	2 mg of carbaryl bran bait (2 % a.i.) in 1.5 ml of sugar solution	Nesting period and progeny development	Nesting duration, cell production rate, offspring survival, parental investment (progeny adult weight), progeny sex ratio	No differences were observed in number of days spent nesting, rate of cell production, offspring survival, parental investment. No sublethal effects on *M. rotundata* larvae	*Bees were fed 1.5 mL of a 25 % commercial honey solution containing the a.i. for 4 days. Cigarette filters used as feeder. On the fifth day, all females were marked and released in greenhouse

Tasei et al. (1988)	Deltamethrin *Megachile rotundata*	Contact exposure Adults	♂: < 0.001 µg/bee (LD_1); ♀: < 0.002 µg/bee (LD_1)	Up to 7 days	Delayed toxicity	In laboratory conditions, applications of doses inferior to the LD_1 to males and females resulted in the reduction of life length of bees. Males were more susceptible than females	
Tasei et al. (1988)	Deltamethrin *Megachile rotundata*	Oral exposure Larvae	Four doses: from 1 to 0.001 mg/kg of provision (1–0.001 ppm)*	From hatching to the end of the cocoon spinning	Larva mortality and larval development	When fed on pollen provisions contaminated with 1 mg/kg deltamethrin, 40 % of the larvae could not reach the final stage. With 0.1 mg/kg the same effects were observed at a lower rate. In both cases, surviving larvae which completed their cocoon developed less rapidly than the control	*After natural contamination with deltamethrin, maximum concentration of residues in provisions was 0.01 mg/kg
Tasei et al. (1988)	Deltamethrin *Megachile rotundata*	Topical application/adult females	LD_1: 0.002 µg/bee	Nesting period (about 6 weeks) in glasshouse	Nesting female survival, number of eggs laid and cells built per female	When 0.002 µg of deltamethrin per bee was applied to females they laid 20 % fewer eggs than control females	

Tasei et al. (1994)	Deltamethrin *Bombus terrestris*	Oral and topical application/adults (worker and queen)	Workers: 0.08–0.16 mg/kg (topical); 0.1–0.2 mg/kg (oral) Queen: 0.01–0.2 mg/kg for 5 days	Several weeks	Food consumption, longevity. Development and size of progeny	Topical application: increase in sugar solution intake from 40 % to 100 %. Oral exposure: reduction of food uptake by 47–59 %. No negative effect on lifespan. No effects on the progeny when queens were fed with sugar solutions contaminated by deltamethrin	
Tasei et al. (2000)	Imidacloprid *Bombus terrestris*	Oral (chronic) exposure via pollen sugar solution Adults	D1 = 10 µg a.i./kg in syrup and 6 µg a.i./kg in pollen; D2 was 2.5 times higher in syrup and 2.7 higher in pollen	85 days	Food consumption, survival rate, brood production, larval development duration.	Food consumption was not affected by either dose. Both doses slightly but significantly affected worker survival rate by 10 % during the first month, without any dose–effect relationship. Brood production was significantly reduced in D1. No significant effect of D1 and D2 treatments on the duration of larval development was revealed	

Tesoriero et al. (2003)	**1.** Kresoxim-methyl **2.** Copper oxychloride **3.** *Quassia amara* extract *Osmia cornuta*	Oral exposure Larvae	1 µL/provision of pesticide at field doses: **1.** 14 g/hectolitre(hl) **2.** 100 g/hl **3.** 400 cm^3/hl	From hatching to the end of the cocoon spinning	Larva mortality and larval development	Mortality rate: **1.** Kresoxim-methyl (13.3 %) did not differ significantly from the controls **2.** Copper oxychloride (44.8 %) **3.** *Q. amara* (82.8 %)	
Torchio (1983)	**1.** Trichloforn **2.** Naled *Megachile rotundata*	In field/adults	Field doses: **1.** 1 lb in 5 gallons/acre; **2.** 8 lb/gal in 5 gallons/acre	7 days	Survivorship of nesting females; cell production rate	In both products, the number of cells completed/day was significantly reduced in the treated field beginning 24 h after treatment	

R. TRIGGER VALUES IN BEES RISK ASSESSMENT

Toxicity Exposure Ratio (TER) of < 10 triggering chronic testing in the risk assessment for soil and seed treatments according to the EPPO scheme (Alix et al., 2009b)

In the EPPO risk assessment scheme for bees (Alix et al., 2009b), there is a factor of 10 between acute and chronic toxic endpoints (LD and LC_{50} values) proposed which, according to Alix et al. (2009b), mainly relies on the quotient between acute and chronic LD/LC_{50} values for bees published in a report by DEFRA (2007). This report provides comparisons of chronic (10 days) LC_{50} concentrations with acute (48 h) LD_{50} doses for seven substances (the first seven compounds in the additional table at the end of this appendix). The authors state an apparent correlation and suggest a 10-fold adjustment factor.

Thompson (2010) added values for further 12 active substances (other compounds in additional table) and plotted the data on logarithmic scales (Figure R1) assuming an adjustment factor of 10 to extend the usage of an adjustment factor of 10 to LD_{50} values between 0.13 and 200 µg/bee. The trendline is $y = 0.893x - 0.817$ ($R^2 = 0.7$).

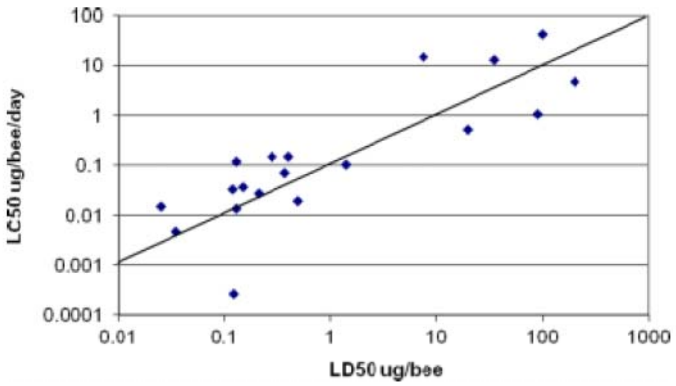

Figure R1: Correlation between 48-h LD_{50} and 10-day LC_{50} data for a range of pesticides in honey bees (Thompson, unpublished data). According to figure 2 of Thompson (2010) plotted on logarithmic scale.

The dataset from Thompson (2010) (see additional table) was re-evaluated. To do so, it was assumed that the ratio between LD_{50} and LC_{50} (per active substance) follows a log-normal distribution. Neither the Shapiro–Wilk test ($p = 0.47$) nor any other goodness-of-fit test was able to identify a significant deviation from this hypothesis. In addition, graphical representation of the data did not indicate a deviation from the assumed distribution (Figure R2). It was further assumed that the literature sample of 19 active substances is representative of the whole set of all active substances. Quantiles of the distribution were estimated by fitting log-normal distribution to the data and estimating them using the fitted distribution (see Table R1). All estimations were done using the Statistical Analysis System (SAS) Version 9.2, especially the UNIVARIATE and REG procedures.

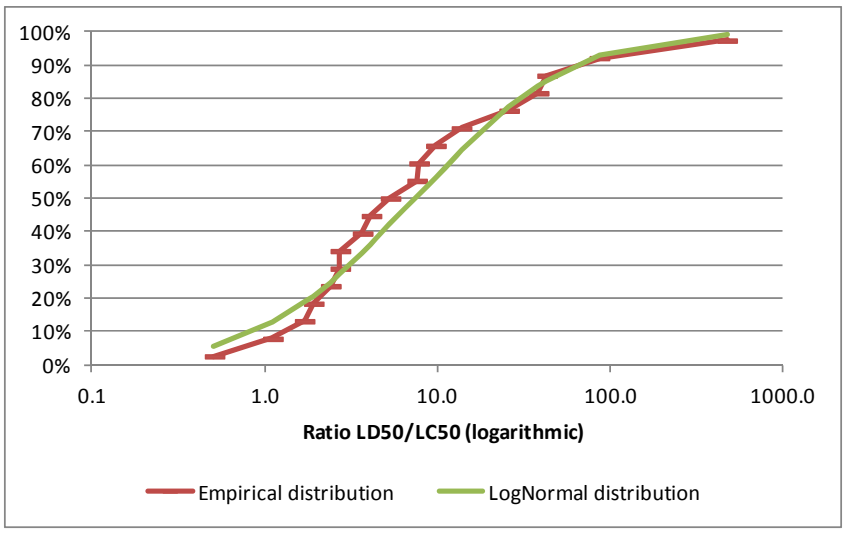

Figure R2: Empirical distribution of logarithmic LD_{50}/LC_{50} ratios from Thompson (2010) compared with the log-normal distribution.

Table R1: Estimated percentile ratios of LD_{50}/LC_{50} with 95 % confidence levels using parametric (log-normal) estimation (Mu = 2.010, Sigma = 1.667).

Percentile	Parametric estimator	95 % Parametric confidence intervals	
		Lower	Upper
95 %	115.800	45.4	579.4
90 %	63.170	27.1	250.7
75 %	22.960	10.8	65.2
50 %	7.460	3.3	16.7

The observed ratios show a median of 7.460 (95 % CI 3.3–16.7) and 90 % of all ratios are below the value of 63.170 (95 % CI 27.1–250.7).

It was estimated from the log-normal distribution that the adjustment factor of 10 from Alix et al. (2009b) is the 57th percentile of the ratios of all active substances.

To estimate the distribution of the extrapolation factors, a sample of 19 substances was drawn from literature. Uncertainties exist if this sample is representative of the total set. Additional statistical uncertainties arise from the statistical estimation process. Higher percentiles, e.g. 90 %, cover main part of the variation and might be preferred. The statistical uncertainty of the estimation can be taken into account by using the upper level of the 95 % confidence interval.

For regulatory purposes, the distribution of extrapolation factors has to be reduced to a single characteristic value which is related to the desired level of protection. It should be decided which estimate (median or 90th percentile with or without CIs) provides an adequate level of protection as an extrapolation factor from acute to chronic toxicity. In order to make this decision, it needs to be taken into account whether the 19 substances in the dataset in Thompson (2010) are representative of all active substances that should be covered by such a factor and whether sublethal effects are also covered in this approach.

First-tier Hazard Quotient (HQ) of ≥ 50 triggering semi-field studies according to the EPPO scheme (Alix et al., 2009b)

In the EPPO risk assessment scheme for Bees (Alix et al., 2009b), there is a HQ trigger value of HQ ≥ 50 proposed triggering semi-field studies. The approach has been used since the implementation of 91/414 (EEC).

Aldridge and Hart (1993) did the first analysis of bee incident data to validate the HQ trigger of 50 for spray products in the EU. This analysis was revised by Mineau et al. (2008) using a larger dataset from the UK. They collected data on hive poisoning incidents reported in the UK's Wildlife Incident Investigation Scheme (WIIS) for the crops oilseed rape and pulses in the UK as well as pesticide use data (area treated and application rate) and data on the physico-chemical properties of the pesticides. They used these data to predict the probability of the detection of a bee field incident by the UK WIIS scheme. To do so, he used a best predictor model including the parameters crop, area treated and $HQ^{contact}$ for all HQs where these data were available assuming a unified area treated for each pesticide–crop combination and concluded from his results that "there seems to be negligible risk" from pesticides with $HQ^{contact}$ values < 50.

Thompson and Thorbahn (2009) used bee incident data from the UK, Germany and the Netherlands to validate the HQ. They collected bee incident data from the three national schemes, calculated HQs using the highest application rate for which data were available in the UK and compared HQs with total numbers of incidents recorded for each active substance. The authors concluded that "the HQ approach to risk assessment for honey bees offers an appropriate level of protection", i.e. in view of their results they do not see a need to change the previous proposal of an HQ of 50.

In the Bees opinion the magnitude of effect has been defined. It has been suggested that the magnitude of effect be quantitatively defined as a certain increase in mortality rate (percentage of bees in hive) over a defined period of time for honey bees but also for other kinds of bees such as solitary bees or bumble bees.

The previous HQ validations (Aldridge and Hart, 1993; Mineau et al., 2008; Thompson and Thorbahn, 2009) were performed using data on reported honey bee poisoning incidents (i.e. records of dead bees at the hive that could be related to pesticide application) for which the magnitude of effect is unknown.

These validations also solely rely on honey bee poisoning data, and it is not clear whether the same HQ can be used for solitary bees or bumble bees. The definition of an incident is that dead bees are found and not a specific number so it may be only a few bees, hundreds, thousands or tens of thousands in one colony or it may be several colonies.

There are some general uncertainties that arise from incident data:

- It mainly depends on beekeepers' willingness and the reporting system. For example in Germany samples can be sent and will be analysed free of charge but not in Switzerland. There is a possibility that Swiss beekeepers will not report each incident because of these charges.

- The probability of noting an incident would also be determined by beekeepers' activity. In spring it is likely they visit colonies/apiaries weekly but later on in the season it might be less often.

If there is an incident recorded and different active substances are found in the dead bees, expert judgement has to be used to find out which active substance is responsible (a causal relation will sometimes be difficult to prove).

Therefore, in the calibration exercise there are many sources of uncertainty that have to be taken into account when validating trigger values.

The validation of the trigger values should ensure that the specific protection goals (SPGs) are assessed correctly at all stages of the tiered Risk Assessment.

For doing such a calibration process correctly it is perhaps necessary to validate the approach by performing semi-field or field studies with pesticides with different modes of action at varying application rates. Regulatory semi-field and field studies are usually performed only if the HQ exceeds the trigger. Therefore just using this database might not be enough. For honey bees in chapter 5 several recommendations are made for improving semi-field and field studies. In addition studies might be necessary to match this approach for honey bees with solitary bees and bumble bees.

Until (semi-)field studies are available to validate the HQ in link with the SPGs, field incident data should be preferred to pure expert judgement.

Data used for such a validation should:

- cover all crops where bees might be affected;
- include soil and seed treatments;
- be representative for all regulatory zones;
- be statistically sound to ensure with sufficient certainty that no incident will be reported below a certain HQ. Important points here are that the effect of pesticides that kill the bees in the field before they reach the hive might be underestimated by bee incident data. Additional variables, e.g. the area on which the pesticide is applied and the crop (see Mineau et al., 2008), might bias the probability of observing an incident and should be therefore taken into account when evaluating the incident data.

If there are incidents for a certain active substance reported and it cannot be proven otherwise, the HQ has to be seen as breached and should be reset.

Additional Tables and Figures

Additional Table: Background data from Thompson (2010, figure 2) concerning TER Trigger of 10 from acute to chronic exposure

	Acute	Chronic	Quotient acute/chronic
	LD_{50} (µg/bee)	LC_{50} (µg/bee/day)	LD_{50}/LC_{50}
Dimethoate	0.13	0.0133	9.8
Deltamethrin	0.21	0.0269	7.8
Pirimicarb	19.5	0.508	38.4
Chlorpyrifos methyl	0.15	0.0362	4.1
Imidacloprid	0.49	0.0189	25.9
Fipronil	0.123	0.00026	473.1
Imazalil	90	1.043	86.3
Oxamyl	0.28	0.1458	1.9
2,4-D	100	41.5102	2.4
Furathicarb	0.13	0.1166	1.1
Emamectin	0.025	0.0147	1.7
Thiamethoxam	0.035	0.0046	7.6
Parathion	0.12	0.0326	3.7
Aldicarb	0.4	0.1461	2.7
Tefluthrin	1.4	0.1019	13.7
Formetanate	0.37	0.0693	5.3
Thiacloprid	35	12.801	2.7
Dodine	200	4.6569	42.9
Acetamiprid	7.5	14.6401	0.5

GLOSSARY / ABBREVIATIONS

a.i.	active ingredient
a.s.	active substance
Apenet	National Italian Bee Monitoring Network
AP-I-PC	*Apis*-In-field-Pollination Crop plants
AP-O-PNC	*Apis*-Off-field-Pollination Non Crop plants
AP-IO-HP	*Apis*-In and Off-field-Hive Products
AP-IO-GEA	*Apis*-In and Off-field-Genetic resources Education Aesthetic values
BBCH	Growth stage; uniform coding of phenologically similar growth stages of all mono- and dicotyledonous plant specie
BR	Biological Response
BROWSE	Bystanders, Residents, Operators and WorkerS Exposure models for plant protection products
CA	Concentration Addition
CLO/CTN	Clothianidin
CEC	Critical Electrolytes Concentration
DAR	Draft Assessment Report
DLR	Dienstleistungszentrum Ländlicher Raum,
EBI	Ergosterol Biosynthesis Inhibitor
EC50	Concentration required killing half the members of a tested population after a specified test duration
EcR	Ecdysone Response
ECx	Concentration with x% level of effect compared to the control
ECx	Concentration with x% level of effect compared to the control
EPPO	European and Mediterranean Plant Protection Organization
ERC	Ecotoxicologically Relevant type of Concentration
ETR	Exposure toxicity ratio

EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FOCUS	FOrum for Co-ordination of pesticide fate models and their Use
GC	Gas Chromatography
GD	Guidance Document
GLM	General Linear Model
Guttation	Appearance of drops of xylem sap on the tips or edges of leaves of some vascular plants
HQ	Hazard quotient i.e. the quotient of the application rate and the acute oral or contact toxicity.
IA	Independent Action
ICPBR	International Commission Plant Bee Relationship
IGR	Insect growth regulator, group of compounds that affect the ability of insects to grow and mature normally
IMD	Imidacloprid
JKI	Julius Kühn-Institut
Lab	Laboratory
LAVES	Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Bieneninstitut Celle
LC	Liquid Chromatography
LC50	Concentration required killing half the members of a tested population after a specified test duration
LD	Lethal Dose
LD50	Dose required killing half the members of a tested population after a specified test duration
LOD	Level Of Detection
LOQ	Level Of Quantification
LTZ	Landwirtschaftliches Technologiezentrum Augustenberg
LWG	Bayrische Landesanstalt für Weinbau und Gartenbau

NAP-I-PC	Non-*Apis*-In-field-Pollination Crop plants
NAP-O-PNC	Non-*Apis*-Off-field-Pollination Non Crop plants
NAP-IO-GEA	Non-*Apis*-In and Off-field-Genetic resources Education Aesthetic values
NBU	National Bee Unit
NOAEC	No Observed Adverse Effect Concentration
NOAEL	No Observed Adverse Effect Level
NOEC	No Observed Effect Concentration
NOEL	No Observed Effect Level
NSA	Non Spray Application
OECD	Organization for Economic Co-operation and Development
OSR	Oil Seed Rape
PEC	Predicted Exposure Concentration
PER	Proboscis Extension Reflex: Pavlovian like reflex in the bee linked to the ability to memorize an odour.
PM10	Particulate Matter ≤ 10 µm
PPP	Plant Protection Product
PRZM	Pesticide Root Zone MOdel
PST	Potter Spray Tower
RA	Response Addition
R&D	Research and Development
RH	Relative Humidity
RT	Residual Time
RUD	Residue Unit Dose
SCFCAH	Standing Committee on Food Chain and Animal Health
SOSR	Summer Oil Seed Rape
SPG	Specific Protection Goal

SSST	Systemic Seed and Soil Treatments
TER	Toxicity Exposure Ratio
THM	Thiamethoxam
TK/TD	Toxicokinetic/Toxicodynamic
TSP	Total Suspended Particulates
TZMU	Thiazolylmethylurea
TZNG	Thiazolylnitroguanidine
UK	United Kingdom
Univoltine	One generation per year
US	United States
WG	Working Group
WOSR	Winter Oil Seed Rape