Environmental Risk Assessment

for Honeybees

State of play and Future Proposals

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CONTENT

NOMENCLATURE

PPP (plant protection products)

SST (Seed and soil treatment)

HQ (Hazard Quotient = application rate/LD_{50})

LD_{50} (Lethal doses 50)

LC_{50} (Lethal concentration 50)

LD_{15} (Lethal doses 15)

LC_{15}c (Lethal concentration 15)

TER (Toxicity Exposure Ratio=LD_{50}/PEC)

PEC (Predicted Environmental Concentration)

PNEC (Predicted No-Effect Concentration)

NOEC (No-Observed Effect Concentration)

NOEL (No Observable Effect Level)

LOEC (Lowest Observed Effect Concentration)

1. INTRODUCTION

Until the early 1990s plant protection products (PPPs) were usually sprayed externally over plants, to kill insect pests; this posed the major risk of acute exposure to pesticides for honeybees. However, the development of pesticide application technologies moved away from externally sprayed PPPs, towards internally distributed systemic pesticides, applied as seed dressings; this systemic technology aimed to confine the PPP concentrations to within the tissues of the treated plant. However, these systemic technologies have radically changed the potential for exposure of non-target vertebrates and invertebrates to these substances.

1.1. The new era of pesticides

The existing Environmental Risk Assessment (ERA) for bees and pesticides, carried out before the introduction into the market of PPPs, was designed in a different era; it was designed for externally applied PPPs that were sprayed onto crops and had no systemic properties. These 'old' PPPs aimed to be short-lived, acute acting and disappeared from the crop within hours or days.

The creation of systemics represent a quantum leap in pesticides - A technological revolution in terms of (a) toxicity (b) persistence in the crop (c) persistence in soil and water (ex. Clothianidin has a half-life of 19 years in clay soils) (d) ability to trans-locate into other untreated crops and wildflowers which are far from the point of application.

This last point means that there is a qualitative difference – in that the old PPPs only contaminated neighbouring crops if the spray drifted on the wind – and even then the toxicity disappeared in a week. The new systemics can migrate via water far away from the field where they are applied and they can affect bees feeding on wildflowers around the edges of the field – or possibly much further away. This 'migration potential' is also a quantum leap in the potential for toxic contamination.

1.2. Bees: social insects

Bees are social insects. Their biology has been adapted to live in well structured societies, in which the relevance of the individual losses importance. Lately, the discoveries of many bee biologists produced a scientific revolution in our understanding of the bee colony – in that it stresses that a colony of bees is really a single super-organism. Despite of the fact that individual bees have a weak immune and detoxification systems for dealing with poisons and pathogens, evolution has sacrificed these systems at individual level for an externalised social immunity. As a result, bees develop several behaviours aiming to keep the colony and its individuals as "clean" as possible. Similar strategies exist for other important functions like thermoregulation of the colony or feeding.

The traditional ERA was designed at a time when the dominant scientific view was that tests should be designed for individual bees. Therefore, the parameters triggering

studies on colonies (through field or semi-field studies) are based on tests on individuals. However, no specifications are done on any effects at social level inflicted on the bee colony by PPPs.

One of the novelties of systemic active substances is that they induce toxic sub-lethal effects (disrupting insects social interactions) even at low concentrations. Indeed, this characteristic is mentioned within the product characteristics and mode of action at the commercial leaflets developed by pesticide producers[1]. Low concentrations of these products " *[…] disorientate the termites and cause them to cease their natural grooming behaviour*." As a result, the product "*[…] makes fungi 10,000 times more dangerous to termites."* This effect that can be observed for target species, works likewise for social benefit species as bees. The long persistence (therefore, toxic action) of the product is also mentioned in this commercial information.

1.3. State of play and existing guidelines to run the ERA

Following Article 4 of the Council Directive 91/414/EEC, Plant Protection Products (PPP) authorised in the EU market must have no harmful effect on human or animal health; nor are they permitted to have an unacceptable influence on the environment (particularly regarding the fate and distribution of the pesticide and its impact on non-target species). As a result, any chemical company that wants to market its products in the EU and to have the active substance included in the authorisation list established by the Directive (Annex I), must submit a risk assessment to the competent authorities, in order for the substance to be authorised at European and National level.

Certain tests are compulsory for any active substance (Annex II of the directive); international guidelines for testing methodologies have been agreed and the European Commission (SANCO/10329/2002) recommends these should be adhered to:

1. Acute toxicity (oral and contact): EPPO Guidelines 170, OECD Guidelines 213 and 214
2. Bee Brood feeding test: ICPBR ((Oomen *et al.*, 1992, for Insect Growth Regulators (IGR)). Moreover, other guidelines exist for the evaluation of toxicity on larvae as the OECD Guideline 75, the test of Aupinel *et al.*, which has been accepted by the French "Commission des Essais Biologiques" in March 2007. In the future, guidelines may also be expected from the COLOSS network.

In addition, PPPs must fulfil further requirements (Annex III of the Directive)

1. Acute toxicity: EPPO Guidelines 170
2. Depending on previous steps, residue tests: No guidelines, but methodologies have been proposed (Lewis et al., 1990) and are currently under development.
3. Depending on previous steps, cage tests: EPPO Guidelines 170
4. Depending on previous steps, tunnel tests: EPPO Guidelines 170

[1] http://www.elitepest.com.sg/brochure/Premise_200SC.pdf

5. Depending on previous steps, field tests: EPPO Guidelines 170

The above guidelines for honeybee risk assessment are validated for the acute effects impacting on honeybees after foliar application of PPPs during flowering (EPPO, OECD). However, although sprayed pesticides only remain toxic to bees for a few hours or a few days, systemic pesticides applied for example via seed-coating and soil-treatments (SST) persist in all parts of the plant for its entire life-cycle; this provides a chronic exposure route to foraging bees and the entire bee colony (Rortais *et al.,* 2005) (see section 2). Plant treatments like: endo-therapy[2], root-baths or applied via irrigation water, distribute PPPs systemically through the entire crop and this needs to be taken into account. The European Commission Guidance Document on terrestrial ecotoxicology recommends the determination of the acute oral toxicity in case PPP application is done as SST. Despite the fact that the same document recommends the measurement of the realistic conditions of exposure (in plant parts), chronic lethal and sub-lethal effects are not evaluated. Consequently the existing guidelines are no longer fit for purpose; they cannot measure or assess the pesticide exposure risk, which systemic PPPs pose for honeybees; in order to address this deficiency scientists have started to develop a variety of new testing approaches.

Many of these new approaches were presented at the Conference of the working group of the International Committee of Plant-Bee Relationship (ICPBR) responsible for the development of these guidelines, in Bucharest (Romania) in 2008. As a result of the Conference, the Working Group of ICPBR submitted their proposal to the European and Mediterranean Plant Protection Organisation (EPPO) in the first half of 2010. The proposal has been published in the EPPO bulletin (Alix and Lewis, 2010).

The present document aims to examine the radically new problems posed by systemic PPPs, when they contaminate bee's food or water sources, thereby creating different routes and patterns of exposure to pesticides. The proposed guidelines will also be reviewed, indicating their limitations and deficiencies when attempting to measure the effects of PPPs. Finally, an environmental risk assessment scheme is proposed, together with proposals to evaluate different effects of PPPs on: bee's survival, their sensory perception and behaviour. Several new concepts are suggested. Those contained in section 4.2 are adaptations of the current guidelines that could lead to an improvement of the ERA in the short term. Furthermore, other suggestions for improvement are included in separate paragraphs, as proposals for major modifications that should be included in the long- term.

2. PATHS OF EXPOSURE

From the mid-1990s systemic insecticides, such as the neonicotinoids and the phenylpyrazoles enjoyed great success, which was equalled by the rapid growth in SSTs. These active substances met criteria specifically tailored to this way of application, namely: they were strongly systemic; they were highly toxic to insects (both pests and non-target insects); they were highly persistent in the plant. As a result, they achieved

[2] Endo-therapy or Phytosanitary Endotherapeutic Injections entails the application of treatments to trees through injection of the active substance or product directly in the tree trunk using systemic products which are transferred by the plant to the core's xylem vessels

market dominance and are used globally for a multitude of crop-applications.

The scientific literature has described new paths of exposure to PPPs coming from SSTs, that differ markedly from the traditionally studied pathways (spray products which imply acute oral and contact toxicity). Some new possibilities of exposure include:

1. The contaminated **dust created during the sowing process** (with pneumatic machinery). The abrasion of treated seeds liberates molecules of these toxic substances into the air, which then contaminate the bodies of forager bees and are thus taken back into the hive (Greatti *et al.,* 2003).

   This exposure pathway was responsible for the serious poisoning incidents which affected up to tens of thousands of hives, in France (2002, 2003), Italy (between 2000 and 2007), Slovenia (2008), Germany (2008) and most likely in many other places from which little information is available. The toxic effects linked to contaminated dust are (sub-) acute in nature. While foraging, the bee is directly exposed to the pesticide particles that contaminate the environment, but she also comes into contact with contaminated surfaces (leaves, flowers, soil, water); in addition she may be affected by the aerosol of toxic particles of dust that she breathes during her flight, or by ingesting food or water contaminated with contaminated dust.

   Some agricultural practices, such as: ploughing, harrowing and straw crushing, also contribute, since they generate clouds of dust as well, which can cause problems when there are high loads of toxic substances.

2. Many published studies have proven the contamination of pollen and nectar with PPPs (ex. Chauzat *et al.,* 2006, Chauzat and Faucon, 2007). **Nectar and pollen** are transported to the hive and stored in wax-cells. Nectar and pollen can be consumed immediately, but the digestibility of pollen is increased after fermentation in storage. Therefore, bees can be exposed to contaminants directly while foraging for pollen and nectar; they may be contaminated by the immediate consumption of these products, or much later, when these products have been stored as the food reserves of the colony. Food reserves are often consumed during the periods of the year when no harvest is possible, particularly during the winter. Therefore, pollen collected in August may only be consumed in March or at the beginning of April in the following year. Consequently, the exposure to pesticide-contaminated food can be delayed in time by 7-8 months.

   The **impact of low doses of pesticides in the nectar and pollen of treated plants** should be examined, in terms of the long-term toxic effects or sublethal effects which may occur, considering that these low doses may be administered repeatedly over a period of many months. Many academic studies are run in confined environments (cage or tunnel) for the development of study protocols evaluating such risks.

   Honeydew is a sugary fluid excreted by aphids as they feed on the sap of a particular crop. If the crop has been treated with systemic PPPs it is likely that the excreted honeydew will contain the active substance. Some may argue that **honeydew** does not pose such a big risk to bees, since any possible systemic

distribution of PPPs within the plant will primarily affect only aphids. However, once bees go to harvest honeydew they may get contaminated with the products/substances present in the environment. In addition, the decrease of aphids in the environment will result in a reduced harvest of honeydew, e.g. Metcalfa in Italy, causing a drop in general food production by the bees, rather than affecting bee health directly (assessed by the ERA).

3. Young plants of almost all crops (from the first unfolded leaf stage mainly to the 6-7 one (BBCH11-BBCH18)) **exude water droplets (Guttation)** during their growing process; it has been scientifically proven that such droplets contain a high load of insecticides (Girolami *et al.*, 2009). These droplet-exudates can eventually contaminate the **morning dew** on the leaves or accumulate in the soil where the crop is planted.

   Although these exudates might not be of interest for the bees from a nutritional point of view (AFSSA, 2009; Thompson, 2010), they cannot be rejected as a drinking water source, as some field studies have demonstrated this use (Riebe, pers. Commun.). Therefore, exudation droplets and dew-water provide two sources of water for insects, together with other superficial accumulations of water at the soil. Further studies are currently under development to determine the degree of relevance of this phenomenon. Moreover, studies developed in the Netherlands have found concentrations of the neonicotinoid Imidacloprid in the **surface water** adjacent to intensively farmed fields, which exceed the maximum allowable risk level of 67 ng/l (Van Dijk, 2010). Since bees undoubtedly collect and drink surface water (rivers, canals, ditches, bunds, etc.), the question to be answered is whether such exposure to contaminated water involves a significant risk.

   Irrigation water used to treat plants and soils with PPPs could also be used by bees as a drinking water source, as well as a source of systemic distribution of PPP throughout the treated plants. Finally, other PPP applications are applied via endo-therapy and root-baths, which involve a systemic distribution of PPPs within the plants.

4. Hundreds of plants (e.g. sunflower) have the capacity to produce **extra floral secretions** that are sometimes intensively foraged by insects (bees, beetles, ants, etc.) under conditions that are still to be determined. Such foraging activity has already been the subject of observations by biologists and beekeepers. Analysis of their composition has established their nutritional value (Mizell, 2009). These secretions that are heavily linked to the sap, are necessarily contaminated with pesticides in the case of SST. However, until now the extent of their contamination by PPPs has never been verified or measured. .

5. Furthermore, the **interactions of the different environmental compartments** need to be taken into account. Leakage of contaminated water or the dispersion of contaminant loads by rain or wind might extend the pesticide exposure possibilities for bees.

6. Poor control of seed coating processes can generate greater or lesser air pollution in the factory where it is made or the field where it is planted.

As a result the exposure of bees to pesticide active substances occurs as follows:

1. Apart from the exposure to toxic particles suspended in the air and pollen, the contact of bees with pesticides is largely through **oral intake** of contaminated food and water sources: nectar and pollen. In marked contrast, sprayed pesticide products mainly affect bees via direct contact toxicity.

2. Contaminated food and water is carried back to the hive by foragers; once these contaminated nutrients are inside the hive, all the bees in the colony will consume them differently, depending on the **caste of bees** (queen, drones, workers) **and the age-class of the bees:** (larvae, nurses, winter bees, foragers, etc) (Rortais *et al.*, 2005).

3. Given the flowering duration of the crop and the possible contamination of the food reserves from systemic pesticides, the **exposure to contaminated material can be prolonged** for many months; in marked contrast, sprayed products are normally degraded faster, principally by light. Such long-term contamination induces a chronic exposure, with lethal or sub-lethal effects, (where no acute mortality is observed, but bees exhibit behavioural abnormalities and health problems).

Acknowledging these paths of exposure, EPPO has published the ICPBR proposal for improving the existing test-guidelines, to better-evaluate systemic products, which may contaminate: pollen, nectar, honeydew and the water sources of the bees. The proposal that ICPBR has submitted is largely an adaptation of the old guidelines established for sprayed products. The following discussion of the current and proposed guidelines highlights their inadequacy in regard to assessing the risk posed to honeybees by systemic pesticides.

3. LIMITATIONS OF THE EXISTING GUIDELINES IN RELATION TO THE ASSESSMENT OF SYSTEMIC PESTICIDES

3.1. Limitations of the EPPO Standards PP3/10 (3)

The EPPO has recently proposed a new Environmental Risk Assessment scheme (ERA scheme) for both systemic and non-systemic pesticides on bees[3]. This aims to take into account the problems linked with the systemic properties of PPPS, through laboratory, field and semi-field testing, as well as honeybee brood testing. However, there is a strong feeling among beekeeping organisations that this proposal still fails to address the risks posed for honeybees by acute and chronic exposure to systemic PPPs; the EPPO proposal is inadequate because, among other factors, it does not evaluate the chronic nature of bees' exposure to systemic pesticides, nor does it address all of the possible exposure pathways (see point 2). It is vital that we achieve the best possible Environmental Risk Assessment schemes, especially at a time when the old regulatory framework is being revised; this is why the following comments have been produced.

[3] December 2010 - http://www.eppo.org/PPPRODUCTS/honeybees/honeybees.htm

Remarks about the proposed risk assessment scheme (original text in annex 1)

- ***Details about the products, patterns of use and possibility of exposure (Points 1 and 2)***

The possibility of bees' exposure to pesticide-contaminated dust and water sources (sap-exudation, morning-dew, superficial or irrigation water or extra floral secretions) should be considered in any new evaluation scheme; these pathways of toxic exposure are not addressed by the current proposal.

It is very difficult to define a list of plant species which are attractive to bees, since this 'attractiveness' is highly variable, depending on the geography, climate and ecology of the different regions of the EU. Moreover, the application of pesticide products has an additional impact on the wider environment of the treated crop (dispersion outside the field via dust, water contamination, soil contamination, etc). Therefore, non-target insects that live on or near such plants, or in the soil beneath, would also be endangered. Thirdly, the evaluation of pesticides is normally assessed using domesticated honeybee colonies, as if honeybees could represent all the pollinator species which are at risk: butterflies, bumblebees, hoverflies etc; but various species of pollinators may have quite different affinities for the same food plants. Scientific knowledge in this regard is patchy and deficient. For this reason it would be unwise, and scientifically illogical, to base the risk-assessment of a PPP and the threat it poses to a wide range of pollinators, on the supposed lack of attraction to bees of the treated plants.

DL_{50} and DL_{15}

The validation of the LD_{50} as 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, given the development of the toxicology since then, other parameters would better assess the toxicity of a contaminant. Moreover, this parameter lacks of a protective dimension since a colony that would loose 50% of its population would find serious difficulties to survive. In fact, some representatives of the scientific community in the field of bee toxicology accept the value of 30% as the maximum of individual loss beyond which the colony is not viable any more. Losses of smaller proportions compromise the activities of the colony, but a healthy colony could survive despite of the fact that its productive yield could be negatively affected. Therefore, estimating for example the DL_{15} would be a much more protective approach both for bees and beekeepers leaving from them. Values of bee mortality under 15% in the essays pose problems since there is a wide variability in the toxicological behaviour of the different PPP. It is of utmost relevance to use the current revision of the EPPO guidelines (for both sprayed products and those applied as seed and soil treatments) to include more modern toxicology techniques and parameters.

- ***First tier test: Preliminary screening based on toxicity (Point 3b)***

Calculate the toxicity exposure ratio between the LD_{50} (oral) and exposure. The new standards propose that if toxicity exposure ratio (TER) between the acute LD_{50} (oral) and the exposure is >10, then the pesticide is categorised as a low risk to bees. Therefore, it is argued that it should not be necessary to perform higher tier tests in order to prove further toxic effects. However, there is a fundamental objection to this approach since TER values <10 are sometimes obtained when highly toxic or widely-spread PPP are considered, those needing further evaluation.

First of all, the deployment of the TER in the ERA entails a negative approach, since it measures the toxicity potential of active substances. Conversely, other parameters could be used which allow waiting for the risk for toxicity to appear; this is a much more positive and protective approach. A good example is the calculation of the HQ (Hazard Quotient) calculated as application rate/LD_{50} (for sprayed products) or PEC/PNEC (Predicted Environmental Concentration/ Predicted No-Effect Concentration). Traditionally, the former has been used for the assessment of sprayed products. As a result, the HQ allows establishing risk coefficients instead of safety margins.

Secondly, if the TER is used following the EPPO proposal, the sentence mentioned in the first paragraph of this section refers to Note 6 of the guidelines, where a DEFRA[4] study is cited on which the proposed safety factor (10) has been based. This study considers that following tests done on 7 substances, the chronic toxicity (LC_{50}, over 10 days) could be derived from the acute toxicity ($LD_{50,}$ over 48h) by applying an adjustment factor of 10 (data shown at annex 2).

This value of 10 should be carefully considered. For example, supposing the acute (oral) LD_{50} of a substance is 5 ng/bee. Following the DEFRA study, the estimation of the chronic LC_{50} would be ≥ 0,5 ng/bee. Following the proposed principle and supposing a bees' exposure of 0,49 ng/bee, the TER calculation would be larger than 10 (5/0,49). Therefore, **the substance or product would be categorized as low risk to bees, even though the bees' exposure would be almost equal to the chronic LC_{50}.**

From the data provided at the DEFRA study (see annex 2) the scheme proposed by ICPBR extrapolates that the chronic LC_{50} is larger than the acute $LD_{50}/10$ ($LC_{50} \geq LD_{50}/10$), for all existing and future substances, and therefore the safety coefficient used with TER should be 10. This extrapolation raises the following concerns:

- From a toxicological viewpoint it is impossible to compare acute and chronic toxicity, since the toxic-kinetics and toxic-dynamics in the individuals exposed to the substances are totally different. For each level of exposure the molecular mechanisms inducing toxicity change depending on the affinity of the toxic for the different biological targets. For instance, the mechanisms, which induce acute toxicity, are not the same to those that induce chronic toxicity (Suchail *et al.,* 2001).

[4] British Department of Environment, Food and Rural Affairs

- The ratio of 10 is not respected for all substances. In the study, for example, Fipronil has an acute LD_{50} of 123 ng/bee and a chronic daily toxicity (LC_{50}/ng/bee/day) of 0,26 ng/bee; in this case, the ratio is 473, which is far from the factor 10 proposed; Besides, each of the 7 substances showed different values for the ratio of acute/chronic toxicity, differing from that (10) in a range going from + 6 (Chlorpyrifos methyl, ratio = 4,14) to – 463 (Fipronil, ratio = 473,08). Even suppressing the possible outlier, the proposed value cannot be obtained.

- Such a generalisation cannot be made just from these 7 substances tested, especially since their range of toxicity varied enormously (e.g. from 123 to 90.000 ng/bee acute toxicity);

- The results of the tests should be questioned since they substantially differ from the data provided at the DAR of some of the active substances studied, e.g. the admitted acute LD_{50} of Fipronil and Imidacloprid are both 4 ng/bee while in the DEFRA study the values are 123 and 490 ng/bee, respectively.

Therefore, the previous points invalidate the EPPO proposal, since the parameter decisions, adopted as a first step to assess the risk category of a

Parameters of used for decision-making

Traditionally the median value of mortality (LD_{50}) has been used for the parameter to measure the toxicity of pollutants. Even in studies of chronic toxicity (over 10 or 11 days) the LD_{50} is used for the expression of toxicity (LC_{50}). Previously, the advantages of the utilisation of the LD_{15} have been presented. However, other parameters like the NOEC (No-Observed Effect Concentration, or NOEL - No Observable Effect Level) or the LOEC (Lowest Observed Effect Concentration) can be used instead of the LD_{50} (either in acute or chronic tests).

PPP have primarily being assessed based on the Hazard Ratio (HQ), ration between application rate and LD_{50}. However other approaches have been described in the scientific literature in order to determine the level of toxicity of a PPP. Depending on the parameter used at the risk assessment, the TER (Toxicity Exposure Ratio), measuring toxicity, or the PEC/PNEC (Predicted Environmental Concentration/ Predicted No-Effect Concentration), measuring protection, different raw data will be needed. The former requests toxicity data to be obtained, while the latter would predict the doses for which no effects are expected based on the NOEL and LOEC. The second approach, the PEC/PNEC, has been suggested as more convenient for the assessment of the effects of PPP in social invertebrates because it enables the protection of the whole colony (Halm *et al.*, 2006). Therefore, future improvements of the ERA can be done on the definition of the parameters and their trigger values used for decision-making.

PPP, are based on an error. The error deepens even further when the EPPO proposal assumes that bees can be taken to serve as 'representatives' of all pollinator species, in the evaluation of PPP. As a result the proposal is inadmissible from a scientific point of view and it should never be applied to the authorisation of pesticides in the EU unless it is thoroughly reviewed and radically revised.

Given the differences observed between acute and chronic toxicity, it is crucial to include the measurement of the latter and link it to the definition of a trigger value, whenever the systemic properties of the product/substance have been determined.

Moreover, the Note states that the concentration in the aerial parts of the plant is overestimated because there is no homogeneous distribution of products and residues (flower barrier). Further up-to-date studies should be presented to support this statement. Existing data about residues are worrying despite their low values. For instance, a concentration of 3 ppb (ng/g) in nectar can result in consumption between 0,24 and 0,96 ng/day/bee (Rortais *et al.,* 2009, forager consumption of nectar between 80 mg and 321 mg) for substances for which the acute LD_{50} remains as well at the order of ng/bee.

Finally, it needs to be reiterated that **plant exudates and morning dew might pose an additional exposure risk, in addition to that posed by superficial water.**

- ***Identification of potential risks for larvae (Point 4 to 6)***

Following Note 7, IGRs[5] have to be assessed performing a bee brood feeding test. Likewise, all systemic substances and products should be assessed through such methods, since nectar and/or pollen can be contaminated with pesticides, thus polluting the colony's food reserves of pollen and nectar, which are the basis for larval food. Moreover, it needs to be emphasized that tunnel-tests might not be the optimal alternative for assessing quantitative effects on bee brood, since a decline in bee brood appears naturally during tunnel tests, as a normal result of bee-confinement. Qualitative effects can be assessed through relevant protocols and parameters that could be prescribed by the guidelines. If tunnel tests are carried out anyway for this purpose, these facts should be taken into account in the guidelines.

- ***Higher tier tests: semi-field and field trials (Points 8 and 9)***

Following the proposed assessment scheme, the sub-lethal effects on bees´ behaviour: (homing flights, bees' locomotion, fertility of drones and queens, etc.) are only assessed through tunnel and field tests. Note 13 says: "[…] *Due to the limitations on replication in field studies and the inherent variability in most of the relevant endpoints assessed, it has to be recognised that statistical analysis may not be feasible […]".* It cannot be acceptable to adopt semi-field and field tests as the only way to assess possible sub-lethal effects, or as the highest tier of the environmental risk assessment scheme, if their statistical validation is not possible.

5 IGR: Insect Growth Regulators

Furthermore, it should be proved in the assessment that bees' exposure did happen and bees should have harvested a normal amount of honey (at least 80% of the average production succeeded on the test crop of a comparable area (e.g. weather conditions, soil, etc.) during the same period of the year). Another approach could be a "temporal control" instead of a "spatial control"; placing the colonies in the fields some time before the treatment is carried out and keeping them there for some time after the treatment could do this. This would reduce test variability arising from colony and field conditions. Naturally, the number of repetitions needs to be large enough to allow statistical validation.

- ***Risk management (Point 15)***

This point proposes risk-mitigation measures, e.g. moving beehives away from crops treated with PPPs. Such measures are often impractical, since many apiaries are static, i.e. the hives cannot be moved; Furthermore, beekeepers are often not notified about the application of pesticide treatments. Unfortunately, long-term experience has shown that even when a product is authorised, with specific precautions for its use printed on the label, these warnings are usually ignored. It should be noted that such a mitigation measure might avoid problem of managed bee colonies. Nevertheless, the risk to wild pollinators would not be avoided.

Therefore, it is arguable that protective measures for bees and pollinators need to be put into place **before** authorisation of PPPs; risk mitigation cannot replace an adequate and complete risk assessment. Therefore, only those substances or products with low risk to bees should be authorised or licensed for use in open field. Moreover, risk mitigation measures should also address the problems faced by the wild - insect-fauna and not just honey bees; these systemic pesticides pose an equal threat to bumblebees, butterflies, hoverflies and a wide range of insect pollinators. Furthermore, only a limited mitigation would be achieved by avoiding pesticide application outside the flowering period, since, if the products are systemic or applied as seed, root or soil treatment, endo-therapy or irrigation water, there is a potential toxic persistence of the products in the soil for months or even years.

3.2. Limitations of the EPPO Guidelines PP1/170(4)

The EPPO has revised its guidelines PP1/170(4) for the side effects of PPPs on honeybees, to adapt them to the specificities of the exposure to PPPs after seed and soil treatments. The guidelines claim that the current assessment of pesticide risk for honeybees, is both robust and effective. However, given the published science, this is only the case for the evaluation of compounds traditionally used as sprayed products; arguably these methodologies are completely inadequate for the evaluation of compounds with systemic properties. In particular, the current HQ (Hazard Quotient) validation includes sprayed applications only (SANCO/10329/2002). Therefore, in an attempt to continuously review and develop the guidelines, certain modifications have already been included. However, since the EPPO standards are still based on the orthodox evaluation of sprayed products, which usually entails only an acute exposure to potential toxic substances, a fundamental change in approach is needed.

In general the guidelines collect acceptable recommendations for the testing of sprayed

products and consider the differences that an evaluation of the effects of products with systemic properties might require. However, proposals for action, to adopt a new methodology which could adequately assess the risks posed by the new systemic pesticides, remain extremely vague.

Remarks about the tests proposed (original text in annex 3)

- ***Comments about the scope of the different tests***

  These guidelines propose methodologies for laboratory, semi-field and field tests. Mortality and acute toxicity evaluation would mainly be assessed in the laboratory. Semi-field/field tests have been proposed to measure: repellence and behavioural effects; effects of persistent residues; effects on aphids, or brood effects; these parameters mainly measure **qualitative** effects. The latter proposals aim to study the effects of PPPs in a simulation of real–world conditions, in case the previous tests indicate a potential risk to bees. This approach, which seems logical for the evaluation of products with a short persistence in the environment, is **entirely inadequate for measuring the effects of products with systemic properties**. In fact, **the duration of the tests proposed in the guidelines is far too short to measure the effects of a chronic or delayed exposure**. Longer periods of study are needed to evaluate: assessment of colony development; delayed effects of contamination; assessment of the exposure to pesticides via the consumption of stored pollen and nectar. Such long-term studies could involve semi-field/field tests, or a combination of both, in which the colony is moved to a non-treated field, where it is kept and controlled for 2 months after the tunnel test.

  Firstly, it is not just **acute mortality** (both oral and contact) that should be measured in laboratory tests; **chronic mortality should also be tested,** as already proposed in various scientific references (Decourtye *et al.*, 2004, Suchail *et al.*, 2001). A methodology has been proposed in paragraph 4.4. Furthermore, although none have been provided so far, guidelines should be devised to achieve the **measurement of sub-lethal effects**. In fact, the French Commission of Biological Essays is considering whether to include tests for sub-lethal effects in its laboratory evaluation of the effects of pesticides. As an example, the effect on the Proboscis Extension Reflex (PER) can be measured in the laboratory. Apart from this reflex, a wide variety of other sub-lethal effects, and methodologies for their assessment, can be found in the scientific literature (and therefore are accepted by the scientific community, see paragraph 4.5). Unfortunately, it takes a long time before a methodology proposed by one laboratory becomes ring-tested. Therefore, in order to investigate the sub-lethal effects of pesticides on bees, some of the studies already published in peer-reviewed journals could be used. At the same time, ring-testing of the available test-methodologies should be accelerated.

  Secondly, certain quantitative effects (in the brood for example) are difficult to evaluate in semi-field tests. If chronic mortality is to be measured through open-air tests, the test duration should be long enough to evaluate the worst-case-scenario, i.e. bees foraging at a treated crop, from seed-sowing to flowering, from harvesting to eventual consumption of stored foods (i.e. the following early

spring), etc.

- ***Remarks on the laboratory tests***

  The guidelines lack to evaluate, in the laboratory, the effects of a possible chronic exposure of bees to PPPs. Instead of counting the number of dead or affected bees at 24h intervals, for up to 48 hours after a unique exposure, effects should be observed for up to 10 or 11 days, following a daily dose of the active substance; at different concentrations (exposure to repeated doses), or following a continuous exposure to a syrup contaminated at different concentrations.

  Following the previous point, sub-lethal effects could also be studied in the laboratory, focusing on behaviours or reflexes crucial to the proper social development of the colony. Based on this kind of test, the PEC/PNEC (Predictable Environmental Concentration/ Predicted No-Effect Concentration) could be calculated and a better evaluation of the impact of the substance could be achieved. For further information on this regard, please see paragraphs 4.2 and 4.5.

- ***Remarks about the semi-field tests***

  Different pesticide active substances have produced different effects, when they were available to the bees on an acute or chronic timescale (Suchail *et al.*, 2001); this is one reason why Dimethoate might not be the best choice as a **toxic standard** (e.g. topic LD_{50} acute is 120 ng/bee; oral LD_{50} acute is 130 ng/bee; oral LC_{50} chronic (over 10 days) is 112 ng/bee/day (DEFRA, 2007). In marked contrast, other substances have proven to have a higher acute toxicity than Dimethoate, and a much larger chronic toxicity, as is the case of Fipronil (measured acute LD_{50} is 4 ng/bee (CST, 2005), measured chronic toxicity is 2,9 ng/bee/day (DEFRA, 2007), NOEC (no-observed effect concentration)<8pg/bee (CST, 2005)) or Imidacloprid (measured acute LD_{50} is 4 ng/bee (CST, undated), measured chronic toxicity is 12 pg/bee/day (Suchail *et al.*, 2001)). These substances could only be used in laboratory tests, since they have been suspended for use in certain Member States (e.g. Fipronil is banned for use on certain crops in France).

Under 1.2 the following is recommended, *"[…] treatments should be applied when the test crop is in full flower except where justified e.g. when recommended product use is pre-flowering."* One should remember that potential exposure following SST, does not only start when the plant blooms, but possibly much earlier, as it grows (through superficial water, exudates, extra floral secretions, air, etc.). Therefore any proposed **test must be capable of monitoring and assessing these routes of exposure; this is one reason why the time of placement of the hive in the treated field, and the duration of the test, should be modified accordingly**. Depending on the scope of the study, i.e. whether it addresses: the risk posed through contaminated nectar and pollen; the risk posed through contaminated water sources, etc., studies will need to be modified accordingly. However, if the latter is extended, it is necessary to consider enlarging the area of the field of study (initially proposed to be 40 m^2).

Toxic standards

Part of the scientific community disagrees with the utilisation of toxic standards. Their utilisation is considered useless since they have totally different physic-chemical properties, metabolism, biological targets, etc. than the molecules of study, reason why they provide no adequate comparison. Besides, the effects of the toxic standard may vary depending on several factors like the health status of the bees, … which makes the comparison useless. As an alternative it has been proposed to control the good health condition of bees and the quality of the solutions used in the study (e.g. purity, concentration, contamination, etc.), together with a larger number of replicates and the development of the test 2-3 times in one year.

The **size of the colony enclosed in the cage or tunnel will depend on the purpose of the study**. The quantity of bees proposed under point 1.3 (3.000-5.000 bees) might be large enough to measure behaviour-related effects. However, this seems too small a colony to measure or assess, for example, quantitative effects on the brood, for which a larger colony would arguably be required (optimal 8 frames with 40.000 bees, or a minimum of 10.000-15.000 bees with 6 frames, 4 of which should contain brood).

Such colonies always include **honey and pollen stores**. One must keep in mind that bees often consume previously stored stocks before the "newly" harvested nectar or pollen. The level of bees exposure during the assay is thus uncertain and should be carefully assessed and proved.

The **number of replications** carried out depends on the relative weight given to

the semi-field trial in the general assessment scheme, but the results should always be statistically significant (at least three replications of each treatment).

Residue analyses might be appropriate for semi-field studies in order to evaluate the potential exposure to PPP.

The EPPO proposal does not include any guideline for the **assessment of delayed effects of pesticide exposure, or for effects over a longer-term period of time**. This could be crucial for the study of parameters like colony development or the possibility of toxic contamination through consumption of stored pollen and nectar. Therefore one option could be: to move the test colonies onto another site, where hives of all treatment groups should be set up together at the same post-treatment location, where no further pesticide exposure is expected (i.e. no/little flowering crops present)' this would avoid any further exposure to different location-specific factors. The harvesting of untreated pollen and nectar from non-crop plants by the test colonies at this stage is avoidable and would reflect normal field conditions.

- ***Remarks about the field tests***

Following the general comment included at the introduction of point 3.2, the proposed guidelines recognise the toxicological differences of systemic compounds from traditionally sprayed compounds. However, no clear recommendations are provided for the evaluation of such systemic compounds, and more specific, targeted guidelines, are needed.

Given the potential exposure pathways described above, the **duration of the trials** needs to be **long enough** to assess whether exposure to pesticides has taken place and if there are noticeable effects, based on the objective of the trial. The EPPO guidelines propose to continue the assessment for longer intervals than 28 days after application, in order to assess colony development. However, delayed effects, or delayed exposure to PPPs from stored pollen and nectar, should also be considered. This could be achieved by taking samples from the food reserves stored in the hive after a long-enough harvesting period. This might require field trials to be extended over several months, depending on the objective of the study.

As with the above comments on semi-field trials, the **size of the colonies** involved in the test depends on the scope of the trial. Point 1.4 proposes the introduction of *"[…] healthy, well-fed, queen-right colonies […] of at least 10.000 to 15.000 bees, according to the season*." This size may be optimal to measure any disruption of behaviours related to foraging, sensitivity to pathogens or bee mortality. However, the study of any effects that would have quantitative impact on food harvesting, or on brood development, would require at least 8-10 frames, 6 of them with brood (minimum approx. 30.000-40.000 bees).

If it is the case that, only laboratory and field tests have been used to assess the risk of a PPP for bees, the **statistical validity** of the latter should be a compulsory requirement.

3.3. Limitations of the OECD Guidelines 213 and 214

These Guidelines are only prescribed for the study of acute toxicity on honeybees, therefore the effects of active pesticide substances, or products with systemic properties, are beyond their scope.

3.4. Limitations of the OECD Guidelines 75

This document establishes guidance for conducting quantitative assessment of the effects of sprayed pesticides, on honeybee brood, under semi-field conditions. Therefore, the tests are **not adapted to the specific duration** of the exposure which occurs after the application of a SST; despite the fact that, either a longer exposure of the hive to the PPP under study, or a lengthier assessment of the effect, could be a better way to adapt the methodology for compounds with systemic properties. Further studies should be carried out to explore the potential of such tests, to evaluate not only bee brood development, but also: sub-lethal effects, bee mortality, delayed effects etc. In such studies it would be interesting to explore the development of different replicates kept in separate tunnels for the entire flowering period of the crop under study, by placing the hives outside the tunnel and controlling them for at least one and a half or two months afterwards.

Following previous comments regarding the **colony size required for the study of bee brood development test** in tunnels, the *"[…] small healthy honey bee colonies (e.g. Mini Plus, nuclei)[…]"* may be too small to measure effects on the brood. Therefore, a hive with a minimum of 6 frames, 4 of which contain brood should be used in the tunnel.

4. PROPOSAL OF ERA FOR SYSTEMIC PRODUCTS ON HONEYBEES

4.1. Introduction

Several sources of pesticide contamination were mentioned earlier, which can affect insects, either as a result of one acute, heavy exposure (see point 2), or via a repeated/constant sub-lethal exposure for more than a year. Such paths of exposure can lead to acute or chronic toxicity, respectively, with effects ranging from actual mortality (acute or chronic) to sub-lethal effects (i.e. behavioural disruptions, loss of orientation, etc). Several proposals for improvement of the current Environmental Risk Assessment (ERA) have been included in this document, some of them suggested as short-term changes, others (included in green paragraphs alongside the text) as recommended future improvements.

Short and long term changes are suggested, due to our serious concern that the proposed adaptations of the existing guidelines (normally used to evaluate sprayed products) to evaluate: seed, plant or soil treatments with systemic products, are plainly inadequate. The current EPPO proposal fails to measure chronic toxicity (over 10 or 11 days); it also fails to address the risks of sub-lethal effects for adult bees (queen and drones fertility, homing flights, bees locomotion, immune capacity…); nor is it adequate for assessing the over-wintering capacity of the tested colonies. Such toxic effects

damage activities which are vital for the development of the colony and its value to the beekeeper, such as: foraging, reproduction, trophallaxy, winter survival, communication, etc. The disturbance of these processes is, *a priori,* unpredictable, hence the reason why the number of tests needs to be statistically significant. There is a need to consider the potential exposure of bees to pesticide-contaminated water and feed sources.

Section 4 aims to update the Environmental Risk Assessment of pesticides for honeybees, with suggestions to correct the inadequacies of the existing standards (see section 3). Our proposals are intended to include all possible sources of contamination and all possible side effects, setting evaluation endpoints/targets. However, these proposals should not be taken as final or definitive: the testing scheme and methodologies need to evolve and develop in parallel to forthcoming scientific findings on the issue. Therefore, future adaptations and refinements should be expected. In order to evaluate the recommended endpoints (for some of which no ring-tests exist so far) and given the long time required for ring-testing of methodologies, we suggest that testers adopt some of the existing methodologies available in the scientific literature (which are already validated by the scientific community) until ring-tests become available.

A new Environmental Risk Assessment Scheme is presented first, followed by other methodological proposals for specific trials.

Bees, between two worlds

Bees are situated in between two worlds: agriculture and environment, as they are environmental food producing animals.

On the one hand, these social insects harvest their feed and water sources everywhere in the environment, reason why they come in contact with any pollutant spread there. Their health and welfare are strongly influenced by the environment around them. On the other hand, bee products are consumed by humans, like any other animal product (e.g. milk, meat, etc). Beekeepers are livestock producers managing their animals (bee colonies, not bee individuals) and producing honey, pollen, royal jell, etc for consumers.

These considerations need to be taken into account when developing a risk assessment of plant protection products on bees (Directive 91/414/EEC). In fact, plant protection products would need to show no unacceptable influence on the environment (Art. 4, 1, b, v), bees included, but also *"[…] no harmful effect on human or animal health, directly or indirectly (e.g. through drinking water, food or feed) […]"* (Art. 4, 1, b, iv).

4.2. A new Environmental Risk Assessment Scheme

Determination of the potential exposure of bees to PPP

Scope: evaluation of the

systemicity and persistence of PPP and the potential exposure of honeybees.

EPPO latest proposal based on Alix *et al.,* 2009, recommends some preliminary considerations as an entry point for the risk assessment that allows framing any potential exposure of bees to PPP. In contrast to Alix's proposal, ours excludes the question about the attractiveness of plants to bees, the main reasons to support our position being: (1) toxins released in the environment can spread everywhere and be present in doses that are dangerous for pollinators, (2) the difficulty of restricting the areas visited by pollinators. Besides, given the possibility of exposure through guttation water shown by certain studies (AFSSA, 2009; Girolami et al., 2009; Riebe, pers. comm.), the present proposal also includes the evaluation of this route of exposure via guttation.

Parallel to the scheme of Alix *et al.,* 2009, the following step considers the **potential systemic properties of the PPPs** (figure 1), regardless of their application pattern. Compounds applied as seed and soil treatments (SSTs), endo-therapy, root baths or through irrigation water, are assessed directly as **systemic**, while the chemical properties of sprayed-products and the analysis of their residues in the green parts of the plants, or pollen and nectar, determine the systemicity of those traditionally applied products. Pesticide-contaminated pollen and nectar brought into the hive by foraging bees constitute a chronic oral exposure pathway. In this respect, Villa *et al.* (2000) proposed to measure the octanol/air or octanol/water partition coefficient (logKoa[6] or logKow) as an indicator of the pollen uptake capability of sprayed products. They also anticipate a methodology that combines application rates and persistence of the active substance in soil (through calculation of DT_{50}[7] and DT_{90}).

Likewise, exposure potential can also be revealed through the **determination of pesticide residues found after treatment**. The residues of active substances and their metabolites (sprayed or applied as SST, irrigation, endo-therapy or root-bathing, in the foraged matrices -pollen, honeydew and nectar - (Alix and Vergnet, 2007) and green plants parts (Alix *et al.,* 2009) some time after the application) need to be evaluated using analytical methods, with limits of quantification of at least the same magnitude as the minimum concentration leading to toxic effects on bees (see point 4.3). Further, limits of detection should be significantly lower than LoQ and should also be applied to all toxic derivatives and metabolites. It should be mandatory to measure potential residues in superficial water that is found around the treated crop. Furthermore, the possible contamination of guttation water droplets from the treated plants needs to be evaluated, as well as the toxic residues they may contain.

Following these preliminary considerations, the potential environmental exposure of bees to PPPs can be categorised, defining the following steps in the risk assessment. If sprayed products do not pose problems of chronic exposure to water and food materials consumed by bees, the evaluation method should conform to the existing standards for sprayed products. In contrast, if it has been proven that food and water sources can be contaminated with the PPP, the evaluation should follow different standards, which take account of a prolonged or chronic exposure to PPP. It is recommended that the acute

[6] Koa: partition coefficient octanol/air. The log Koa is directly proportional to the uptake by leaves of hydrophobic organic chemicals from the air. It is relevant to study those sprayed PPPs absorbed by the plants leaves/pollen as vapour.
[7] DT_{50} is the rate of degradation of pesticides expressed as half-life, in years, months or days. Every pesticide has its own DT_{50} value. After this period only half of the original amount of pesticide is left, the other half having been degraded away. DT_{90} is also a parameter to express the degradation rate, meaning the time needed for the degradation of 90% of the pesticide.

and chronic toxicity of the active substance/ compound should be evaluated, which leads us to the following step in the assessment.

Data obtained at this stage:

- logKoa or logKow
- DT_{50} or DT_{90}
- PEC in each of the materials analysed (superficial water, guttation, pollen, nectar, green parts of the plant…)

Tier 1. Risk assessment of systemic pesticides to adult and immature individuals

Scope: evaluation of the toxicity to adults and immature individuals

Any problems revealed for adult bees or larvae by acute or chronic toxicity tests (see below), would require further studies, in order to evaluate the effects on the colony.

Acute toxicity to adult individuals

A first step in the risk assessment of PPP is to measure the effect on adult honeybees of acute exposure to the compound in question. The toxicity derived from contaminated water sources (if applicable) or foraged matrices is mainly oral. Therefore, if systemic properties have been proven in earlier stages, at this step acute oral toxicity will be assessed. Several methodologies already exist to do this. (EPPO, OECD).

Despite the fact that previously (see green paragraph at 3.1) we have discussed the possibility of including the LD_{15} instead of the LD_{50} in order to increase bee protection, from now on the LD_{50} will continue to be used. In doing so, we maintain the traditional approach (therefore not requiring a re-evaluation of the active substances already on the market) despite the fact that the use of risk-factors becomes necessary.

Acute Reference Dose for bees

The FAO/WHO proposed to measure the Acute Reference Dose (ARfD) of pesticides. As the FAO/WHO (JMPR)[1] stated in 2002 this parameter is "an estimate of the amount a substance in food or drinking water, normally expressed on a body weight basis, that can be ingested in a period of 24 h or less without appreciable health risks to the consumer on the basis of all known facts at the time of the evaluation". The JMPR counts with guidelines to establish this parameter published at *Food and Chemical Toxicology,* 43, 1569-1593 (2005).

This approach could substitute the present toxicity measurement based on the LD_{50}, by the ARfD achieving even a more protective approach.

[1] Joint FAO/WHO Meeting on Pesticide Residues (JMPR)

For all products showing a potential to contaminate honeybees the **Hazard Quotient (HQ = application rate/LD_{50})** can be used in order to determine if further studies should be carried out or not. For sprayed products we propose the traditional approach of HQ>50 requiring further studies. However, for systemic pesticides, further studies in the field or semi-field, will enable us to prove the adequacy of this value, or it may be preferable that scientists working on bee toxicology propose this value.

Conversely, Alix *et al.,* 2009, recommend the use of TER (Toxicity Exposure Ratio, **equal to LD_{50}/PEC**) instead of the HQ and suggest the 90th percentile of the data set of residues for the calculation of the TER at this step, and in case these data are not available, a generic residue value of 1mg/Kg. Similar to HQ, TER values represent the relationship between the toxic effect of the active substance and the degree of exposure suffered by bees. Slightly toxic active substances, or those having only small amounts of residues in the mentioned materials, would be considered to pose a low risk for bees. Otherwise further evaluation would be required. The TER is related to a **security factor** deciding the following step in the assessment (see 3.1). This factor should be high enough to reduce false negatives to a minimum, in order to better protect bees from possible chronic or sub-chronic effects. According to the scientific literature, such a security factor could have a value ranging between 1.000 and 100.000 (Suchail *et al.*, 2001).

The **HQ and TER** do provide information about the active substance, but the heterogeneous behaviour of different active substances makes it crucial to run **chronic toxicity tests, as well as tests on immature individuals, every time an active substance has been shown to have systemic properties**.

Chronic toxicity to adult individuals

To date, no official guidelines have been proposed for the assessment of the long-term effects of PPPs. Point 4.4 proposes a methodology for chronic mortality tests in the laboratory. The existing guidelines for the assessment of acute toxicology have been taken as a basis (EPPO 170 and OECD 213 and 214) for the development of this methodology; however, modifications have been introduced: to increase the period of study and to simulate exposure to contaminated material (Decourtye *et al.*, 2005).

From the results of the chronic mortality test the HQ (**HQ_2**) can be calculated considering the **bee exposure** (PEC, consumed quantities of active substance) **and the PNEC** (Predicted Non effect Concentration)[8]. The determination of the PNEC requires a series of tests: to measure the lowest concentrations at which an effect does appear (LOEC), and the no-observed effect concentration (NOEC). The PNEC is placed between the LOEC and NOEC and can be predicted through a coefficient of security depending on the accuracy and reliability of the tests (Halm *et al.*, 2006). The quantity of the substance consumed by the bee depends on (1) the concentration of the active substance in the pollen and nectar (analysed, (Rortais *et al.*, 2005; Halm *et al.*, 2006)) and (2) the amount of pollen or nectar actually consumed by the bee (estimation: amount consumed through pollen and nectar).

[8] PEC/PNEC is the ratio of the concentration one expects to find in the environment and the concentration that causes no adverse effect to the Environment.

Again, if the **HQ_2 is smaller than a risk coefficient**, then the active substance will be judged to pose a low-risk for bees, while a higher HQ_2 would require further assessment. The value of the risk coefficient will depend on the difference between the chronic toxicity and those doses which produce sub-lethal effects. Scientists working on bee toxicology are best placed to propose such a value.

Risk assessment to immature individuals

The potential exposure of the colony to contaminated material may produce a different impact on larvae than it does on adult bees. Given that the larval food reserves of the colony (pollen and nectar) may be contaminated with pesticides, there is an evident need to test immature individuals (larvae) as first tier.

Various methodologies have been developed to study pesticide effects on brood in tunnel tests (OECD 75; Oomen, P.A., 1992). However, such tests provide only **qualitative** information about the development and health of bee brood. Aupinel *et al.,* 2005, proposed a test to measure lethal effects (both acute and chronic) and larval growth success in the laboratory, using a methodology that permits an isolation of the larvae for the **quantification** of such effects. This methodology has been ring-tested.

With the data obtained from the test, the HQ (**HQ_3 =PEC/PNEC** (for larvae)) can be calculated and a trigger value can be set (e.g. PEC/PNEC <1) depending on the acceptable risk achieved by the evaluation. Alix *et al.,* 2009, propose a safety coefficient of 1 for the TER on brood tests measured from the ratio NOEL-exposure. As this expression of TER is the inverse of the PEC/PNEC, the risk coefficient would be 1. This would mean that if the PEC is larger than the PNEC further tests would need to be carried out to verify the development of the brood in field/semi-field conditions. Again, it would be advisable that the scientific community should agree on a good risk coefficient for the HQ on larvae.

Data obtained at this stage:

- Acute LD_{50}
- Application rate
- Chronic LC_{50} (adults)
- NOEC adults
- PEC adults
- HQ_1 (application /LD_{50})
- HQ_2 (PEC/NOEC)
- LD_{50} and LC_{50} (larvae)
- PEC larvae
- NOEL and LOEC larvae
- HQ_3 (PEC /NOEC) larvae

Tier 2. Risk assessment to the colony

Risk assessment to the social activities of the colony and its duration

Scope: evaluation of sub-lethal effects of adult and immature individuals and development of the colony.

Bees are defined as 'social insects' in that they cannot survive individually and many aspects of their survival, resistance to diseases, etc depend upon communal activities such as grooming, communication, social immunity to pathogens…

Given the importance of social behaviour and activities expressed by the individuals of the colony which are vital for its healthy and correct functioning, it is of utmost importance to evaluate the effect that sub-lethal doses of PPPs could have on such social behaviours.

PPPs have been shown to have sub-lethal effects on pollinators in numerous scientific publications (Desneux *et al.* 2007, Kacimi El Hassani *et al*., 2007, Guez *et al.*, 2001, Aliouane *et al.*, 2008) describing effects on the waggle dance (parathion), the harvest and the transport of nectar (diazon), the homing flight (deltamethrin) (Vandame *et al.*, 1995). Specifically, various deleterious effects have been described on the honeybee: larval development, life span and survival, fertility and egg-laying capacity of

Estimation of future effects on adult individuals

Recent publications have shown the relationship doses-time-effect with regard to certain toxics, insecticides being among them (Sanchez-Bayo, 2009 and Tennekes, 2010). Such relationship that follows the Haber law and known as the Druckrey–Küpfmüller equation, explains why toxic effects may occur at very low exposure levels, linking the dose of a contaminant with the duration of exposure until the outbreak of the toxic effect (the one considered at this stage would be death, however the calculation would apply as well to other toxicity symptoms). Having evaluated both acute and chronic toxicity for each of the active substances, this equation would have an added value in predicting effects of pesticides on non-target insecticides.

The equation is as follows:

$$DT^{1/b} = \text{constant}$$

where "T" the median toxic-effect induction time, "D" the daily dosage of chemical and the exponent "b" is a constant calculated for each substance and each animal species. The value of the constant "b" has already being calculated for various species (*Daphnia magna*, *Cyprodopsis vidua*, *Gammarus pulex L.*, etc). Further studies will allow the calculation of this parameter for honeybees and the different substances in the market. However, this equation has limitations: it cannot be used to make estimations in large variations of time or doses in the long-run because insecticides with systemic properties have an erratic behaviour. All in all, the equation is a very interesting approach that is worthwhile to develop.

the queen; the mobility of the bee; its navigational ability over short and long distances; foraging intensity; feeding behaviour; learning capacity and thermo-regulation. Particular attention should be paid to the effects on the social-immune-system capacity of the colony as a whole, given the importance of certain social behaviours for immunity and resistance to pathogens and parasites. Damaging effects have already been documented for various substances and microorganisms, some non-pathogenic organisms becoming pathogenic when associated with defined substances, e.g. Imidacloprid (Alaux *et al*., 2009). Indeed, the **disruption of individual behaviour** (odour discrimination, recognition of related bees, etc), which indirectly leads to problems at colony level, can be evaluated through some tests. Conversely a **decrease in the social activities of the colony** (contribution of nectar to the hive, brood size, thermo-regulation, etc.) can be evaluated without especially studying the individual level.

Therefore, it seems inappropriate to classify the tests depending on <u>where</u> they are carried out (laboratory, tunnel, cage or open field) as is currently the case in the EPPO Standards, but it is more important to precisely define the endpoints or the methods to study the bee's activities/behaviours, either individually or as a social colony. Regardless of where the tests are developed it is crucial to control the exposure to PPPs in order to determine the toxicological relevance and relative value of the methods used.

Several behaviours or activities can be identified as essential for the health and survival of the colony. The following list is not an extensive list and could be modified depending on future scientific developments: life-span, survival and egg-laying duration of the queen, homing flight, orientation and navigation, feeding of brood, thermo-regulation, learning capacity (Proboscis Extension Reflex), etc.

A precautionary approach (**HQ_4 = PEC/PNEC**) has been suggested, in order to better assess sub-lethal effects, which may occur in social invertebrates such as honeybees (Halm *et al.*, 2006). Point 4.5 proposes an example of a methodology to assess sub-lethal effects on a range of behaviours, from which the PEC/PNEC can be calculated. Despite the fact that the methodology proposed is carried out in laboratory conditions, other tests need to be carried out in semi-/ field tests (e.g. homing fly, thermo-regulation, etc). However, none of these methods has so far been ring tested.

Rortais *et al.,* 2005, highlights the differences between the various castes and classes of bees, which could be crucial at the time of deciding which individuals should be tested. The PEC is based on measurements of the active substance and its relevant metabolites in the foraged matrices. This parameter can be established for different types of bees (males, workers, queens and among the workers: nurse bees, foraging bees), which allows us to quantify the variable exposure for different categories of bees.

As mentioned before, the PNEC is placed between the LOEC and NOEC and can be predicted through a coefficient of security depending on the accuracy and reliability of the tests (Halm *et al.,* 2006). These tests should be framed/ targeted for the specific category of bees and for a specific behaviour. Various methods appear in the literature, which allow us to determine the PNEC. Many of them are already used in labs: bee-brood-feeding test (Aupinel *et al.*, 2007), bee-locomotion, learning ability assessed through the proboscis extension reflex (Decourtye *et al.,* 2004), bee thermo-regulation. However, there are other behaviours that can also be assessed in the field or by semi-

field tests: homing flight (Vandame *et al.,* 1995, Bortolotti *et al.*, 2003), foraging intensity (Giffard and Mamet, 2009; Colin *et al.,* 2004). The life-span of the bees, the egg-laying capacity of the queen and the fertility of drones should also be assessed, since these capacities are crucial for the survival of the colony. The robustness of the methodology is not always tested and some of these should be validated in different laboratories to achieve ring tests that could be brought into the assessment scheme.

Therefore, if no significant effects are observed in colony development and the calculation of the PEC/PNEC for the different activities, or the behaviours in question, is in accordance with the trigger value established (e.g. PEC/PNEC < 1), then the product will arguably not pose a risk to bees. A committee of scientific experts on bee toxicology could determine the trigger value for HQ.

Data obtained at this stage:

- NOEC and LOEC
- HQ_4 = PEC/PNEC for different behaviours
- Overall development of the colony

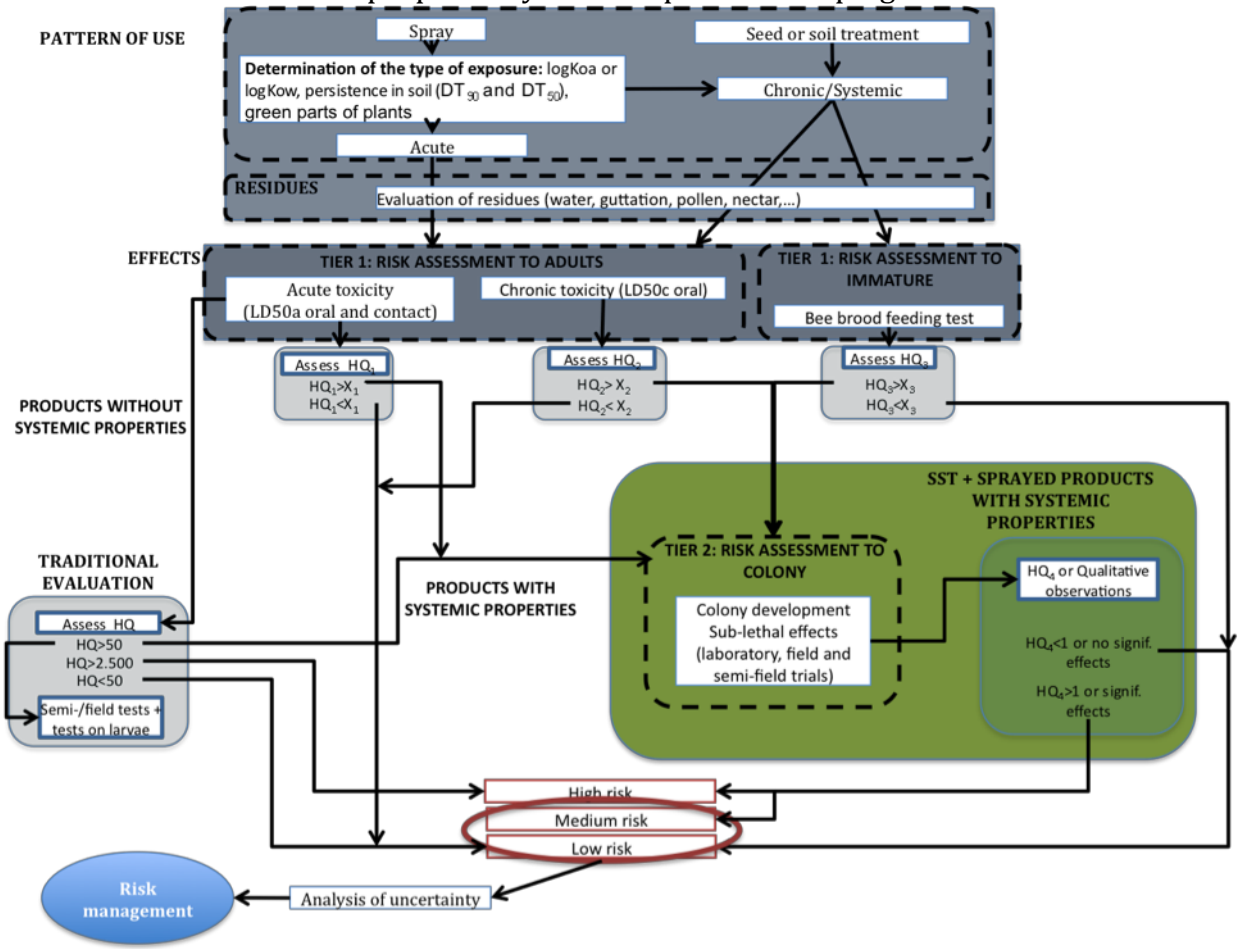
Analysis of uncertainty

Following on from this risk assessment scheme, an analysis of uncertainty needs to be completed in order to consider other factors like calculation errors, amount of data available, etc. For this step the approach shown at the EPPO standards PP 3/10 (2003) should be taken.

Monitoring of residues

It would be good practice to monitor the long-term persistence of toxic residues for some years after a newly authorised PPP is introduced to the market. Such an approach would encourage better control over the impact of the product in real-world conditions and would make the regulatory process more transparent. EU Member States should be currently developing methodologies for residue monitoring, following the provisions included at Commission Directive 2010/21/EU of 12 March 2010 amending Annex I to Council Directive 91/414/EEC as regards the specific provisions relating to clothianidin, thiamethoxam, fipronil and imidacloprid.

Figure 1. Environmental Risk Assessment Scheme proposed by the European Beekeeping Coordination



4.3. Methodology proposed for the assessment of residues in the foraged matrices

The analytic methods used should be able to detect the concentration at a comparable level to the NOEC or LOEC over a long period of time, for active substances that are toxic at these levels. That is to say, active substances that are toxic at higher doses (e.g. DDT), do not need such low values of detection; while in contrast, Imidacloprid, which has a NOEC of only 200 pg/bee would need to be measured at far lower limits of detection- as little as 0,1 ppb (CST, 2005).

A limit of quantification (LoQ) of the order of ppb (ng/g) should be recommended for all active substances measured in complex matrices (i.e. pollen and nectar). Liquids allow us to achieve far lower limits of quantification (LoQ), picograms/g . Such minute quantification limits are possible nowadays and methods for achieving them are already described (Bonmatin *et al.,* 2005).

In respect of pollen, the current analytic method must include the dissolution, or complete grinding-up of the pollen envelopes, because the toxic substances are found inside the pollen grain and not on its surface. Pollen should be sampled from pollen traps on the hive, or preferably from the actual flowers foraged by the bees, since this is the pollen they have consumed. Bee-bread can as well provide precious information about the active substances that the colony will consume in reality. Trapped pollen from mixed crops needs to be analysed and identified in order to estimate the contamination level for a specific crop. Pollen stored in the comb is derived from a mixture of different plant-sources. This should be considered when sampling pollen and evaluating conclusions.

Residue detection and quantification

The limits of detection and quantification have evolved historically thanks to the development of analytical techniques and methodologies. Indeed, many of the problems shown by bees and other insects in the past found an explanation as soon as it was possible to detect lower doses of pesticide residues in the environment. This is the reason why further techniques need to be explored in order to achieve even finer limits of detection. Such limits could eventually get to NOEC levels, which would mean a step forward of the Evaluation Risk Assessment.

The quantification of PPP residues in nectar also requires clarification. Nectar collected by foragers and brought back to the hive is metabolised by worker bees, using enzymes from their digestive system; the treated nectar is then deposited in wax cells and reduced by evaporation to as little as 20% of its original volume, when it becomes known as honey. During this enzyme-metabolism, PPP residues may be metabolised by the bees, therefore the concentration of residues found in the original nectar may be modified. Therefore, we should consider the differences that may exist between the quantification of residues in raw nectar (taken for example from flowers or foragers) and in the final honey which is stored in the hive.

4.4. Methodology for the determination of chronic toxicity

4.4.1. Experimental conditions

- **Principle of the trial**

  It is logical to focus on **oral toxicity** when assessing bees' chronic exposure to PPPs, since the normal pathways of pesticide contamination are via bees´ food. Consequently, oral toxicity of test compounds for adult worker honeybees is assessed in the laboratory. Bees are exposed to continuous but different amounts of the compound by feeding variable doses. Mortality values are used to provide a regression line and LC_{50}.

- **Trial conditions**

  Bees are kept in holding cages that are well ventilated and easily cleaned. Plastic cages should not be used, unless they are disposable, because of possible contamination. Re-use of wooden cages should be avoided unless they are very well cleaned and sterilized. Cages should not cause control mortality though contamination.

  Since temperature is a parameter which causes variation in the effectiveness of substances/products, the assays should be done at three different temperatures, one simulating spring (approx 15ºC), another at average temperatures (approx. 25ºC) and another which simulates the temperature conditions of nurse-bees or summer conditions within the hive (approx. 35ºC). Relative humidity during the test should be kept constant and recorded. Bees should be kept in darkness for the whole trial period, except during assessments.

- **Preparation of the bees**

  Young adult worker bees of similar age should preferably be used (preferably newly emerged bees). Bees should be adequately fed and should be taken from a healthy and queen-right colony. Where applicable, the time of the last Varroa treatment should be identified and recorded. The treatment should have ended at least 4 weeks before the start of the test. Bees should be collected in a standardized way. Collection in early spring or late autumn should be avoided. Bees may also be reared in an incubator, fed with fresh or well-preserved pollen (not contaminated and varied) and sucrose solution (of organic origin). The method of collection used, the age and (if known) the race of bees, and date of the experiment should be recorded.

- **Design of the trial**

  Treatments: either formulated products or active substances are tested. A control treated with the dosing vehicle should be included. Regarding the use of a toxic standard to check consistency of results, two options are proposed:

  - Use of toxic standard: In the case of systemic PPP the frequently used toxic standard Dimethoate (Gough *et al.,* 1994) is not the best option since other active substances exist with larger chronic toxicity as already

mentioned above. Therefore, other active substance with high chronic toxicity should be taken as toxic standard, e.g. Imidacloprid (LC_{50} = 12 pg/bee/day (Suchail *et al.,* 2001) in contrast with LC_{50} 112 ng/bee/day for Dimethoate (DEFRA, 2007)). Toxic standards should be bought from enterprises specialising in analytical standards. Otherwise, the purity of the toxic standards should always be verified prior to running the tests.

- No use of toxic standard: In this case the quality of the bees and of the fabrication of the toxic solution should be assured and the number of replicates will be increased (4-6 replicates). The same test will be repeated 2-3 times in the year and the toxicity of the product will be obtained at the end through statistical analyses.

Test units: bees should be dosed individually or in groups of at least 10. They should not be confined individually for more than 1 h.

Replicates: at each concentration, at least three groups of 10 bees should be used. For limit tests, the number of groups should be increased to 5.

Concentrations: formulations of sugar syrup (of organic origin in order to avoid contamination) with different concentrations of compound and offer it permanently to bees during the trial period. A suitable range and number of concentrations should be used in order to provide a regression line, NOEL and LC_{15}.

4.4.2. Application of treatments: Oral toxicity test

- **Test product(s)**

The formulated product or active substance should be used in a 50% w/w final concentration of sucrose solution. Formulations should be dissolved or dispersed without additional solvents if possible (but if these are necessary, the solvents should be administered to the control at the same concentration).

- **Mode of application**

A dose of 10 or 20 µL of test solution per bee should be supplied through single-use feeders each morning for 10 days. The length of the trial should not exceed 11 days, since bees do not tolerate such lengthy confinement. Through group-feeding, bees share the test solution among themselves and so receive similar doses. There should be a maximum period between dosing (e.g. 4-6 h) to avoid deaths from starvation.

If at the end of this period, there is still some test-dose solution remaining, the amount should be measured. This allows the precise dose taken by the bees to be determined, which is more accurate for the LC_{15} calculation and provides information on the distastefulness or actual repellence of the active substance in solution.

Fresh sucrose solution (of organic origin) should be provided after the dose has been taken; this should be refreshed daily.

4.4.3. Mode of assessment

The number of dead or affected bees should be counted at 24-h intervals during the length of the trial (additional assessments at shorter intervals may be useful in specific cases).

4.4.4. Results

Tests should be repeated if the control mortality exceeds 15%. Mortality should be assessed after correcting for control mortality. Appropriate statistical methods should be used to analyse the results and calculate the lethal dose value (LD_{15}), expressed in µg of active substance per bee and/or µg product per bee (when conducting the risk assessment both exposure and toxicity should be expressed in terms of active substance or product).

If the ratio dose/mortality curve shows irregularities or variations in trial replications, the safety factor adopted should be higher than the one normally used.

4.4.5. Accumulation potential

By calculating the Chronic toxicity/Acute toxicity ratio (LC_{50}/LD_{50}) the potential accumulation of a substance can be assessed. If this ratio is greater than 2, this indicates that the sensitivity to repeated doses is more than double the sensitivity to a single dose, confirming a clear cumulative effect. In such case, a greater safety factor should be used for the TER calculation.

4.5. Proboscis Extension Reflex (PER)

The test of the proboscis extension reflex (PER) is used to evaluate the bees ability to memorise an odour.

- **Principle of the test**

The bee is held in restraints (harnessed) which only permits free movement of the head (antennae and mouthparts). A device offers several floral essences or other odours (pheromones, for example) to the bee; one of these odours is associated with a sweet tasting reward (syrup supplied on a cotton swab). The bee undergoing the trial quickly associates the odour with the sweet reward, pushing out its tongue (proboscis extension) whenever

the odour is present, but not when it is absent. The PER test measures the number of trials it takes before the bees to learn to associate a specific odour with a reward (i.e. it tests acquired memory) or to forget the association (i.e. this tests the extinction or erasing of memory).

- **Biological Significance**

  Bees can recognise a wide variety of smells. This ability is vital to foraging performance, since the different levels of acquired memory (short, medium or long term), enable the bee to navigate easily from one flower to another; it also enables the bee to store information about interesting flower patches between successive flights and to remember nectar sources from one day to another. The bees' ability to wipe and re-set navigational memory, allows it to exploit successive nectar sources throughout the season, as their nectar production rises or falls.

  However, the PER does not enable us to account for all the foraging activity of bees, since they use a complex system involving both behavioural patterns (visual recognition of shapes and colours) as well as their ability to discriminate and choose between variable sugar levels in different floral nectars. Nonetheless, memory capacity as assessed by the PER, is central to success in foraging (Menzel R. , 1999).

  Odour discrimination also plays a key role in regard to the social immunity of the colony. In particular, the bees are able to sense a specific odour through the opercula (wax capping) of sick brood; when they detect such a warning odour, they bite through the wax cap to extract and destroy the infected larva. Some genetic strains of bee exhibit enhanced sanitary (hygienic) behaviour in comparison to other strains; these 'hygienic bees' demonstrate a higher sensitivity to the smell of contaminated brood, which stimulates them to remove the infected larvae before the entire hive is infected (Gramacho and Spivak, 2003). In similar manner, some strains of bee are able to detect the odour of varroa mites within a sealed brood cell; such bees actively destroy the parasitized larva and all the contents of the cell – thus reducing the level of varroa infestation. Consequently, the PER is a crucial indicator for behavioural patterns that are vital to the survival of the hive.

- **PER in the scientific literature**

  The PER test is not new. It is based on the discovery in 1957 of a Pavlovian reflex in the bee (Kuwabara *et al.,* 1957). Nowadays, it is widely used by researchers on bee neurobiology. The PER has allowed us: to model the neural reflex (Menzel and Giurfa, 2001); to characterize the different levels of memory in the honeybee (Menzel R., 2001); to measure the effect of sleep-deprivation on the memory capacity of the bee (Hussaini *et al.*, 2009); to assess bees ability to discriminate between comb-waxes of differing age (Fröhlich *et al.,* 2000) or to discriminate healthy brood from sick brood (Gramacho and Spivak, 2003). Various laboratories have already used the PER test to quantify the effects of sub-lethal contaminants (Decourtye *et al.,* 2005, Guez *et al*., 2001, Bernadou *et al.*, 2009). There is a broad peer-reviewed literature on PER and it is widely used by leading

researchers from various institutes including: the Freie Universität Berlin, the Laboratory of Ethology and Animal Cognition of the University Paul Sabatier at Toulouse, the institutes of Ecophysiology and Sociobiology of the University of Würzburg, the laboratories of INRA Avignon and Bures-sur-Yvette, the laboratory of CNRS Bordeaux, etc.

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7. ANNEXES

ANNEX 1. EPPO Standards PP3/10 (3)

Organisation Européenne et Méditerranéenne pour la Protection des Plantes
European and Mediterranean Plant Protection Organization

10/15949 for comment

Normes OEPP
EPPO Standards

PP3/10(3)

Environmental risk assessment scheme for plant protection products

Chapter 10: Honeybees

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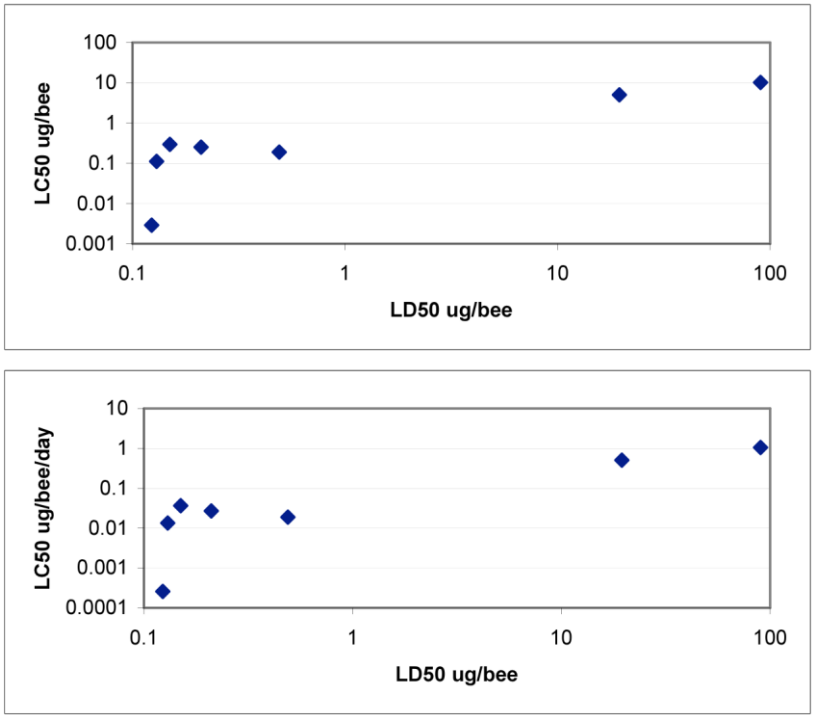
ANNEX 2. Results of Defra study, 2007

Table 1. LD_{50} (48h) and LC_{50} (10 days) for pesticide active ingredients offered in 50% w/v sucrose.

	LD_{50} ng/bee	LC_{50} accumulated ng/bee	LC_{50} ng/bee/day	LD_{50}/LC_{50}
Dimethoate	130	112	13,3	9,77
Deltamenthrin	210	253	26,9	7,81
Pirimicarb	19 500	5 010	508	38,39
Chlorpyrifos methyl	150	293	36,2	4,14
Imidacloprid	490	189	18,9	25,93
Fipronil	123	2,9	0,26	473,08
Imazalil	90 000	10 245	1 043	86,29

Note: Highlighted in yellow those active substances that would be assessed as low risk for bees, even though some of them are highly toxic when the chronic toxicity is measured.
Source: DEFRA, 2007

Figure 1. Comparison of the LD_{50} and LC_{50} (both daily and accumulated) for the 7 pesticides assessed



ANNEX 3. EPPO Guidelines PP1/170(3)

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

PP 1/170(4)

Efficacy evaluation of plant protection products

Side-effects on honeybees

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