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P.A. Oomen, H.M. Thompson (Editors)

Hazards of pesticides to bees

10th International Symposium of the ICP-BR Bee Protection Group

Bucharest (Romania), October 8-10, 2008

Julius Kühn-Institut
Bundesforschungsinstitut für Kulturpflanzen

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INTERNATIONAL COMMISSION FOR PLANT-BEE RELATIONSHIPS

BEE PROTECTION GROUP

10th International Symposium

HAZARDS OF PESTICIDES TO BEES

Bucharest, Romania

October 8-10, 2008

Organised by

Beekeeping Association of Romania
Institute for Apicultural Research & Development, Romania

Plant Protection Research Institute, Romania

Place

The Symposium was held at the

Conference Hall of the Romanian Beekeeping Association,

42 Blv Ficusului, 013975 Bucharest, Romania

Organising Committee

Pieter A. Oomen (PPS, NL), Chairman
Helen Thompson (CSL, UK), Secretary
Gavin Lewis (JSC International Ltd, UK), Vice-Chairman
Dietrich Brasse (Braunschweig, D), Vice-Chairman
John Stevenson, (Harpenden, UK)
and
Cristina Mateescu (Institute for Apicultural Research & Development, RO)
Carmen Mincea (Institute of Plant Protection, RO)
Cristian Constantinescu, (FIITEA of Apimondia, RO)

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ICP-BR

The International Commission for Plant-Bee Relationships (ICP-BR) was founded in 1950 by the Swiss scientist Anna Maurizio whose outstanding work was mainly devoted to bees and their relationships with plants. Since 1980 this commission – which is affiliated to the International Union of Biological Sciences (IUBS) – has regularly organized in Europe working sessions on the harmonization of methods for testing the toxicity of pesticides to bees.

ICP-BR develops the scientific process preceding decisions from European administrative authorities, EPPO (European and Mediterranean Organisation for Plant Protection) and OECD (Organisation for Economic Cooperation and Development). ICP-BR Bee Protection Group symposia have acquired considerable authority in the area of legislation and regulation concerning bee protection related to the use of plant protection products, bringing together the European expertise of national authorities, industry and research.

The Bee Protection Group held its first meeting in Wageningen in 1980 and over the subsequent 29 years has become the established expert forum for addressing the risk of pesticides to bees. It has operated by reaching consensus amongst a wide range of experts active in this field drawn from industry, regulatory authorities and research institutes across the European Union (EU). Operating through the EPPO honey bee sub-group, it has produced the testing methodology and risk assessment guidance currently used under Directive 91/414/EEC.

ICP-BR symposia Honey Bee Protection

1st symposium	Wageningen NL	1980
2nd symposium	Hohenheim D	1982
3rd symposium	Harpenden UK	1985
4th symposium	Řež CZ	1990
5th symposium	Wageningen NL	1993
6th symposium	Braunschweig D	1996
7th symposium	Avignon F	1999
8th symposium	Bologna I	2002
9th symposium	York UK	2005
10th symposium	Bucharest RO	2008

Symposium excursion

Symposium excursion to the Romanian-Orthodox Snagov Monastery

School of Environmental Sciences, University of Guelph

Guelph, ON N1G 2W1, Canada

Foreword

The Bee Protection Working Group of the ICPBR has, for three decades, provided an important forum in which representatives from industry, national and international regulatory agencies, government and academic research bodies, and others can come together to address and assess the hazards to bees and pollination posed by crop protection operations. Such meetings bring the diversity of concerned parties into collaborations that help ensure that the common and sometimes divergent interests of crop production, beekeeping, pollinator management, agriculturally based industries, researchers, and conservation of sustainably managed and natural ecosystems are aired productively and objectively. Each time the group has met, it has provided crucial guidelines and advice. The 10th meeting, held in Bucharest, is no exception.

I am honoured to have been asked to write this Foreword to this latest volume, and to extend congratulations and thanks to all those who contributed to the meetings in its initiation, organization, and execution, to those whose participation and discussion were essential, to those who wrote up their findings and conclusions, and to the editorial team who brought this important and timely publication to fruition.

It is important to acknowledge the supporters of the 10th meeting, so special thanks are extended to the Romanian Beekeeping Association, Apimondia Romania, the Research Institute for Plant Protection in Romania and the international companies sponsoring this meeting.

The importance of this book is amply illustrated through the overarching and global concerns of *The Convention on Biological Diversity* that recognizes that pollinators and pollination are crucial to global productivity in agriculture and nature and are under serious environmental stresses. The Bee Protection Working Group of the ICPBR is a vibrant and effective model that offers world leadership. I am sure that the excellent work will continue as the environmental stresses facing bees, other pollinators, pollination, agriculture, forestry, human life, and natural systems become more complex and global in scope. Certainly, it is important to take the present model as it pertains especially to the European & Mediterranean Plant Protection Organisation (EPPO) to the world.

Peter G. Kevan, Ph. D., FRES, FIBiol
Chairman, ICPBR & Scientific Director, Canadian Pollination Initiative

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I. Regulatory issues (incl. Revision of EPPO risk assessment and guidelines)

II. Test and risk assessment (incl. systemic effects, field testing, bee brood)

III. Bumble-bees and other bee species

IV. Test methodology (laboratory, cage, field, sub-lethal, etc.)

V. Honey bee poisoning incidents and monitoring schemes

Introduction to the symposium

Pieter A. Oomen, Chairman ICP-BR Bee Protection Group & Editor

Some facts about the Bucharest symposium

The 10th International Symposium, Hazards of Pesticides to Bees, of the International Commission for Plant-Bee Relationships (ICPBR) Bee Protection Group was held at the Conference Hall of the Romanian Beekeeping Association, 42 Blv Ficusului, 013975 Bucharest in Romania on October 8th to 10th 2008. The 79 participants were welcomed by the Chairman of the Bee Protection Group, Dr. Pieter A. Oomen. The president of the Romanian Beekeeping Association Dr. Eugen Zorici, the Director General of the Research Institute for Apiculture Dr. Petre Moraru, both the hospitable hosts of the symposium, also welcomed the participants. Prof. Horia Iliescu, director of the Plant Protection Research Institute, opened the symposium on behalf of Dr. Elena Leaota, director of the Phytosanitary Agency of the Ministry of Agriculture of Romania. The chairman warmly thanked these hosts of the symposium for their hospitality and cooperation. On behalf of the ICP-BR Bee Protection Group he also thanked the sponsors of the meeting:

- BASF Ag
- Bayer CropScience AG
- Dow AgroSciences
- E.I. Dupont de Nemours
- Syngenta Ltd.

The chairman then introduced the working group board members and organisers of the symposium: Helen Thompson – secretary, Gavin Lewis – vice-chairman, Dietrich Brasse – vice chairman, and himself, Pieter Oomen – chairman. They were effectively supported by the local organisers Cristian Constantinescu – director general of Apimondia Romania, Cristina Mateescu – Research Institute for Apiculture, Carmen Mincea – Research Institute for Plant Protection.

Why ICP-BR, why this 10th Symposium, why in Bucharest, Romania?

Chairman Pieter Oomen explained the role this ICPBR working group has played in the last 30 years in Europe. The Bee Protection Group is formed by scientists committed to the safety of honeybees and other bee species in agricultural crops and to ensure that they are not harmed by the approved use of plant protection chemicals. The Group fully accepts the need for agricultural chemicals and the vital part they play in efficient food production. The ICP-BR Bee Protection Group held its first meeting in Wageningen in 1980 and over the subsequent 30 years has become the established expert forum for addressing the risk of pesticides to bees. It has operated by reaching consensus amongst a wide range of experts active in this field drawn from industry, regulatory authorities and research institutes across the European Union (EU). Operating through the EPPO honey-bee sub-group, it has produced the testing methodology and risk assessment guidance currently used under Directive 91/414/EEC. It remains a scientific conscience of the effectiveness of the honey-bee risk assessment and risk management in the EU by monitoring and discussing effects and recommending solutions to EPPO, OECD and EU. In this 10th symposium, also a delegation from the Belgian and French beekeepers associations participated, who presented her view on effective honey bee protection.

In view of new developments on honey-bee protection in Europe and the world, e.g. suspicions about the possible causes of so called colony collapse disorder, bad overwintering successes of colonies, pesticide caused incidents in different countries, the European and Mediterranean Plant Protection organisation EPPO had required thee ICP-BR Bee Protection group to recommend about the updating of the current risk assessment approach and initiate the necessary preparations. This request was an important reason to organise this symposium.

This 10th meeting was organised in Romania in order to involve also the new EU member countries, and because of Romania's recent history with beekeeping, Apimondia activities, twinning activities, good facilities and enthusiasm among the Romanian colleagues to host this meeting. Also the chairman's recent activities in Romania as RTA (as Resident Twinning Adviser during 2006 and 2007 in the Romanian Ministry of Agriculture) had been another reason.

About the programme

The programme of the symposium existed of sessions on (a) Tests and risks assessment, (b) Bumble bees and other species, (c) Test methodology, (d) Regulatory issues, (e) Excursion to the interesting Snagov area with informal dinner, intended to enable participants to establish personal contacts and networks, (f) Plenary discussion for revision of the EPPO guideline and risk assessment scheme, and finally (g) Reports about honey bee poisoning incidents and monitoring schemes, this year with particular attention for actual incidents caused by dust abrasion from treated seeds in Germany, France, Italy and Slovenia. And of course there was a poster session on these subjects.

The essentials of the work of ICP-BR Bee Protection Group, both past and current, are summarised by vice chairman Gavin Lewis in his presentation.

New in this symposium was the presentation of a beekeepers view upon risks and risk assessments in Europe by a representation of Belgian and French beekeepers association. Their extensive view is published in these proceedings.

Major attention in this symposium was given to the results of the three different working groups, established at earlier symposia, notably York 2005, in order to solve the recently emerged problems of systemic effects through seed and soil treatments, of semi-field and field testing, and honey-bee brood testing. Their proposals were plenarily discussed in order to hear the expert comments and recommendations of the whole symposium and to take profit of the 30 years of accumulated expertise. The working group coordinators would elaborate their group's proposals with these comments and recommendations, after which these proposals will be offered to EPPO as recommended material for an updated EPPO scheme for testing and risk assessment of pesticide effects on honey-bees.

The presentations and proposals presented in the different sessions are reproduced in these symposium proceedings as paper or abstract of presentation by the respective authors. The plenary discussion is fully described by the secretary Helen Thompson.

During the symposium, Dietrich Brasse from Germany announced his departure as vice chairman of this Bee Protection Group. His departure was regretted but his contributions during many years were very much appreciated, in particular his promise for this symposium to publish the proceedings in the Julius Kühn Archive. His successor, also from Germany, will be Ingo Tornier of EuroFins Agroscience Services. The symposium very much welcomed Ingo Tornier as new vice chairman.

Conclusions

Major results of the meeting were the reports of the three working groups, established at the previous symposium (2005) in York UK. Working groups are an established tool of the Bee Protection Group, by which issues that are identified at the meetings as requiring further work are taken forward by volunteers representing industry, regulatory authorities and research institutes. The three working groups made recommendations in the regulatory review: systemic effects of seed and soil treatments, bee brood effects and field-testing as well as the bee brood ring-testing group. In addition, the bumble bee working group had continued its work and collected further data that will allow a comparison to be made between honey-bee and bumble-bee susceptibility to pesticides. All working groups presented their findings for consideration by the whole meeting. These proposals were discussed and agreed upon by nearly all representatives at the symposium. Only the representation of Beekeepers Associations from Belgium and France, led by Janine

Kievits, made reservations. Their views, however, extensively published further in these proceedings, are being taken as source of inspiration by the working groups in their further elaboration of the proposals.

The recommendations of the working groups record the views of the majority of the 79 experts present at the meeting in Bucharest. These experts represent the accumulated knowledge and experience gained in the Bee Protection Group over the last 29 years and of the government departments, beekeeping organisations and commercial companies represented by the many members of the Group.

The Bee Protection Group will offer the recommendations of the working groups to EPPO, and assist in developing these into an updated risk assessment scheme for pollinators, for better protecting the honey bees, bumble bees and other pollinators from the negative effects of plant protection products. This way the ICP-BR Bee Protection group will continue to provide its expertise to EPPO, EU and other interested organisations.

The assessment of pesticide risk to bees: the work of the ICPBR 'Bee Protection Group'

Gavin Lewis, Vice-chairman of ICP-BR Bee Protection Group

JSC International Ltd, Simpson House, Windsor Court, Clarence Drive, Harrogate, HG1 2PE, UK
Phone: +44 (0)1423 520245, Fax: +44 (0)1423 520297, E-mail: Gavin.Lewis@jsci.co.uk

Abstract

The 10th Symposium of the International Commission for Plant-Bee Relationships (ICPBR) Bee Protection Group was held on 8-10 October 2008 in Bucharest, Romania. A major part of this meeting was given over to a revision of the EPPO guideline 170 and the associated risk assessment scheme, which forms the basis of regulatory evaluations for the effects of pesticides on honey bees in the EU. While the current EU risk assessment scheme is considered to be robust and effective, such revisions are considered appropriate as part of an ongoing process of review and appropriate development. The revision process was based on reports presented by three working groups that had been set up at the 9th Symposium of the Bee Protection Group (York, UK; 2005). The three groups had addressed the following issues: (1) higher tier testing (cage and field trials); (2) the risk to bees from the use of plant protection products through seed coating and soil applications (systemic effects); (3) the risk to honey bee brood (including *in vitro* larval testing methodology). These proceedings present the current proposals for the revised EPPO honeybee testing guidelines and risk assessment scheme. These will be subject to a final review before being submitted to EPPO and also to EFSA for consideration as part of the revision of the Terrestrial Ecotoxicology Guidance Document.

Keywords: risk assessment, honey bees, guidelines, revision

Introduction

The 10th Symposium of the International Commission for Plant-Bee Relationships (ICPBR) Bee Protection Group was held on 8-10 October 2008 in Bucharest, Romania. This group is the European expert forum addressing the risk of pesticides to bees, representing academia, regulators and industry. There were about 80 delegates from 15 European countries present at the meeting. A number of papers were presented, addressing a range of issues including test methodology, honey bee poisoning incidents and monitoring schemes, the risk to bees from insecticidal seed treatments and bumble bees. In particular, a major part of the meeting was given over to a revision of the European Plant Protection Organisation (EPPO) honey bee test guidelines and risk assessment scheme. This paper provides a brief introduction to the Bee Protection Group and provides the background to the work that was done in relation to the assessment of pesticide risk to honey bees.

EU Regulatory Risk Assessment

The ICPBR Bee Protection Group provides the technical input to the EPPO 170 guideline[1] and associated risk assessment scheme[2]. This in turn currently forms the basis of regulatory evaluations for the effects of pesticides on honey bees in the EU[3]. In addition, more recently, the EPPO 170 guideline has formed the basis of the OECD laboratory test guidelines for acute contact and oral toxicity to honey bees (OECD Guidelines Nos. 213 and 214 [4,5]).

The approach to honey bee risk assessment that has been developed has proved to be robust and effective but at the same time it is recognised that a continuing process of refinement and development is appropriate to ensure that the guidance is clear and responds to any concerns identified during use. Accordingly, a review was carried out in 1999 at the 7th symposium in Avignon, France[6] and this resulted in the current versions of the EPPO guideline 170 and the associated risk assessment scheme[1,2]. More recently, EPPO had asked the ICPBR Bee Protection Group to undertake a similar exercise at the 10th symposium in Bucharest.

Honey bee risk assessment scheme

Honey bees have been the subject of regulatory data requirements at the national level in the EU for more than 50 years. Initially, the assessment comprised toxicity classifications based on laboratory generated data but it soon became clear that in many cases this was not a good indicator of effects seen in the field. This resulted in the development of the hazard quotient, one of the first occasions there had been a consideration of the relationship between toxicity, as measured in simple laboratory assays, and exposure under field conditions[7]. This was subsequently incorporated into a sequential testing scheme [8] that forms the basis of the current approach to risk assessment for honey bees and other non-target groups.

The sequential risk assessment scheme incorporates different levels of testing into a stepwise procedure. This starts in the laboratory with the simple acute contact and oral toxicity tests and where appropriate is followed by testing with increasing levels of realism and complexity i.e. semi-field (cage) tests and full field studies. The assessment of the data produced by this testing is risk based and at Tier 1 this involves the use of the hazard quotient, the ratio between the application rate and the toxicity (LD_{50}) value. Based on a comparison of HQ values with the known risk to bees for registered compounds in the Netherlands, a threshold value was set at 50[8]. Below this level it is considered that there will be an acceptable risk to bees i.e. there will be no effects when plant protection products containing a particular compound are used under field conditions. HQ values greater than 50 indicate that there is a potential risk and that the significance of this cannot be ascertained without additional, higher tier data. This threshold value of 50 has been validated using incident scheme data from a number of EU countries and has been shown to provide an appropriate level of protection[9].

The higher tier testing incorporates increasing levels of realistic exposure into the testing. Thus, at the semi-field level free-flying colonies of bees are confined in mesh covered cages over plots of the test crop that is treated in a manner reflecting normal agricultural practice. In full field studies, honeybee colonies are placed adjacent to large plots of a test crop e.g. a standard attractive crop such as *Phacelia* or the crop relevant to the intended use of the plant protection product. In both cases, a range of assessments are carried out including mortality, behaviour of the bees on the crop (foraging activity) and at the hive and the health of the colony (including brood assessments). This more complex data set is inevitably difficult to interpret in terms of considering the significance of any effects seen as well as assessing the overall impact on colony performance and thus requires a degree of expert judgement.

In addition to the core scheme as outlined above, a number of additional aspects can be taken into account in the current honeybee risk assessment scheme. Thus, it may be appropriate to consider the duration of any residual toxicity e.g. when considering safe intervals before exposing colonies to treated crops. However, although test methodology is available none has been validated for regulatory use and at the last guideline revision[6] it was agreed that this should only be an optional requirement. Specific effects may be identified in the initial testing and investigated further e.g. repellency and synergism. Particular attention is paid to compounds with insect growth regulatory activity, with a specific test method available for assessing effects on bee brood[10]. In the final assessment, it may be necessary to impose risk mitigation measures to demonstrate acceptability and while general guidance is given on this, it is recognised that this must be implemented at the national level taking into account local conditions, agricultural practices etc.

Revision process

The Bee Protection Group is keen to promote national incident schemes and one reason for this is that they can identify issues arising from actual use that may require further consideration within the risk assessment process. While the current EU risk assessment scheme is considered to be robust and effective it is also recognised that a continuous process of review and appropriate development is necessary. This needs to be done in a considered way with the development of a consensus view amongst the expert representatives within the group. This allows any new information to be evaluated and its significance in relation to the risk for honeybees assessed. This is the approach that has been adopted by the Bee Protection Group in both the previous revision in 1999 and has been used in the current revision requested by EPPO.

The current revision process was based on reports presented by three working groups that had been set up at the previous meeting of the Bee Protection Group (in 2005 at the Central Science Laboratory, UK)[11]. These working groups are used to address in detail specific issues identified at the main meetings and then report back at the next symposium in order that their proposals can be discussed and a consensus view obtained. In this case the three groups had addressed: (1) higher tier testing (cage and field trials); (2) the risk to bees from the use of plant protection products through seed coating and soil applications (systemic effects); (3) the risk to honey bee brood (including *in vitro* larval testing methodology).

1. Concerns had been raised that systemic activity is not adequately addressed by the conventional regulatory risk assessment for foliar applied pesticides. This relates to the exposure of bees from soil-applied pesticides (seed treatments, etc) that move through a plant into flowers, nectaries and aphid honeydew. While this issue is considered to some extent within the current EU risk assessment scheme, it was considered that its potential significance might require a separate-harmonised risk assessment scheme. This would comprise a similar sequential or step-wise design that would identify the circumstances in which information on systemic activity is required and determine how it should be used within an assessment of risk, including identifying appropriate trigger values for higher tier assessment.

2. Currently, EU regulatory requirements for honey bee brood are addressed by the acute toxicity testing in adults together with the initial risk assessment using the hazard quotient. Where higher tier testing is triggered, brood effects are taken into account according to the semi-field and field test guidelines. Only in the case of insect growth regulatory compounds (IGRs) are there specific testing requirements, which currently follow the EPPO guideline[10]. However, this methodology has never been validated and there have been reported problems with its reproducibility. A new *in vitro* test is being developed[12], which assesses toxicity to bee brood primarily via the oral route of exposure. Consideration has been given to incorporating the toxicity data produced into the risk assessment scheme, taking into account brood exposure and again identifying appropriate trigger values for higher tier assessment.

3. In addition, a working group was set up to review the current guidance for higher tier testing i.e. semi-field (cage) test and full field studies. The aim of the EPPO 170 test guideline is to provide sufficient guidance to allow the studies to be conducted without being too prescriptive. It was considered that this should be looked at again in the light of experience obtained with the working of this guideline over many years. In particular, it was recognised that developments in the other working groups highlighted the fact that higher tier testing might be triggered via a number of different routes e.g. adult toxicity, brood effects, systemic activity etc. Accordingly, it is important that the guidance is sufficiently detailed and flexible to address the different emphasis that each requires.

After receiving presentations from the working groups, a plenary session of the 10th Symposium discussed the proposals in detail in order that the consensus view of the meeting could be obtained. The resultant reports of the working groups are presented in this volume[13, 14, 15]. This will now be taken forward into specific proposals for revised test and risk assessment guidelines by the EPPO honey bee sub-group during 2009.

Conclusions

This paper presents the current situation with regards to the proposed revision of the honeybee testing guidelines and risk assessment scheme. However, it was agreed at the 10th Symposium that revised versions of the Bee Protection Group's proposals, incorporating the comments received at the meeting as appropriate, would be circulated to all delegates for a final review. The Bee Protection Group proposal for the revision of the honey bee test guidelines and risk assessment scheme will then be submitted to EPPO during 2009. They will also be sent to the European Food Standards Agency (EFSA) for consideration as part of the revision of the Terrestrial Ecotoxicology Guidance Document.

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I. Regulatory issues (incl. Revision of EPPO risk assessment and guidelines)

Guidance for the assessment of risks to bees from the use of plant protection products applied as seed coating and soil applications – conclusions of the ICPBR dedicated working group

Anne Alix*, Marie P. Chauzat, Sophie Duchard, Gavin Lewis, Christian Maus, Mark J Miles, Ed Pilling, Helen M. Thompson, Klaus Wallner

*AFSSA-Direction du Végétal et de l'Environnement, 10 rue Pierre Curie, 94704 Maisons Alfort Cedex, France
Phone: 33 1 49 77 21 26, Fax: 33 1 49 77 21 50, E-mail: a.alix@afssa.fr

Abstract

Background: Soil or seed applied plant protection products (PPPs) aim at bringing the amount of active substance involved to the only parts of the plant that have to be protected. Despite a reduced exposure of non target organisms by this way, an exposure of honey bees through residues in pollen and/or nectar may not be excluded for substances that migrate towards the upper plant parts. Directive 91/414/EEC, related guidance documents and literature data were reviewed and discussed by a working group of the ICPBR (International Commission for Plant-Bee Relationships) with the aim to provide adequate guidance to proceed in a risk assessment in such cases.

Results: The review and expert knowledge collected within ecotoxicology, entomology and plant residue area allowed to identify the key parameters that trigger a risk assessment as well as basic hypotheses to consider in deciding for the experimentations required (laboratory, semi-field and field tests). A stepwise, tiered approach is proposed, which has been checked for its ability to discriminate substances that may pose a risk to bees from substances of low concern.

Conclusion: The present scheme is proposed to update the current EPPO risk assessment scheme with a special issue on systemic PPPs.

Keywords: risk assessment, honey bees, soil or seed treatments, systemic.

Introduction

The Plant Protection Products (PPPs) through seed coating or soil applications on bare soils are intended to concentrate the product in/on the plant parts to be protected, and/or to areas where pests are the most abundant. Exposure of non-target organisms is reduced compared to spray applications, as it is intended to be restricted to the area where the organisms are living in the soil and potentially to vertebrate species that feed from these soil organisms.

An exception to this however may occur when products display systemic properties, as in this case growing plants may contain residues. Exposure of bees may then arise if substantial amounts of residues reach flowers, and particularly nectar and pollen which constitute bee food resources.

Directive 91/414/EEC and related guidance documents do not provide detailed technical guidance on how to proceed to assess the risks to bees posed by substances with systemic properties.[1, 2, 3] This issue was debated at the ICPBR (International Commission for Plant-Bee Relationships) meeting in York in October 2005,[4] and a working group was constituted with the aim to identify the key issues for a new risk assessment and to propose some guidance on a harmonized risk assessment scheme at the European level.

This paper presents the approach to develop this risk assessment scheme followed by the ICPBR working group. Based on a detailed analysis of the conditions for exposure of bees to residues, the scheme proposes a stepwise approach starting with simple calculations based on existing data available in the authorisation dossiers, and ending with field studies. Every assumption is discussed in the light of the review of available data in the fields of bee ecology, ecotoxicity and chemistry of PPPs in relation to expected levels of residues

in plants. The resulting risk assessment scheme has been tested with a data package for PPPs of different categories in order to check whether it discriminates between low risk and high risk products.

Current Regulatory Background (Directive 91/414/EEC and related guidance documents)

Directive 91/414/EEC identifies the conditions for use of PPP for which the exposure of bees cannot be excluded, namely systemic seed dressings, systemic preparations for application to soil and systemic dipping treatments for transplanted crops and bulbs.[1] The relationship between systemic properties and the exposure lead to emphasis on the decision making criteria (annex VI of Directive 91/414/EEC) "where relevant, any information on the persistence of residues in the treated plants". However, little recommendation on how to assess the residues content in the treated plants and to deduce exposure levels is given. The guidance document on terrestrial risk assessment recommends to perform an acute oral toxicity test on bees with the active substance in all cases where a product is to be applied as a soil/seed treatment and involves a systemic substance.[3] Then for substances for which a risk is identified at this stage ("e.g. very low LD_{50}"), it is proposed to "take into account realistic exposure conditions, as for example exposure concentrations as expected in nectar and pollen as indicated by residue studies". Nevertheless, no other indication is provided to trigger this step but it is recommended that exposure, to which the oral LD_{50} (lethal dose 50) could be compared, should be "expressed based on the compound (active substance or metabolite) present in the respective plant parts (e.g. nectar, pollen) to which honey bees could be exposed". The next step, triggered by a risk at this stage, would be to envisage "higher tier studies (cage/tent/tunnel or field studies) with realistic exposure scenarios". Current recommendations thus quite quickly refer to higher tier studies, mainly because "estimates of the concentrations of compounds in the relevant plant parts are rarely available" and "exposure calculations in higher tier studies are already considered within the experimental design (e.g. honey bees foraging on treated field crops)".[3]

Decision making criteria are commonly defined for all PPPs whatever the mode of application, by considering that "where there is a possibility of bees being exposed, no authorization shall be granted if the hazard quotients (HQ) for oral or contact exposure of honey bees are greater than 50, unless it is clearly established through an appropriate risk assessment that under field conditions there are no unacceptable effects on honey bee larvae, honey bee behaviour, or colony survival and development after use of the plant protection product according to the proposed conditions of use".[1] It is acknowledged however that a critical HQ of 50 was validated against field studies with sprayed products which is therefore relevant for sprayed products,[3,5,6,7] while of that kind there is no validated decision making criteria applicable to a first step risk assessment for non-sprayed compounds. Practically, in the absence of clear criteria on the level of concern raised by a LD_{50} value, to assess the exposure of bees and the potential risks, the evaluations that have been proposed in dossiers mainly follow case-by-case approaches and thus miss the normal harmonisation for usual risk assessments in the regulatory context.[1]

Exposure of bees including hive bees to products applied as soil/seed treatments

Conditions for exposure to residues from soil/seed treatments and for risks to the colony

Basically, the exposure of bees to residues of a product applied for crop protection as a soil/seed treatment may occur if the following three conditions are met: (1) the plant is visited by bees; (2) there is a transfer of residues, either the active substance or a degradation product, from the seed or the soil to the upper part of the plant and to plant matrices of interest to the bees (pollen, nectar, honey dew); (3) for an exposure at the scale of the colony or population scale, contaminated matrices are brought back to the hive by foragers and consumed by the colony. For a risk to occur in the colony from the exposure to these residues, the level of exposure needs to be higher than the threshold for effects, as defined from laboratory and/or higher tier (semi-field, tunnel, field derived) data.

In order to provide detailed recommendations when deciding whether bees can be exposed to residues under specific conditions for use of a PPP, a review of the literature was performed on the issues of (1) the attractiveness of plants to bees; (2) typical level of residues to be expected in plants, and matrices such as

nectar and pollen from systemic transfers; (3) the predictability of systemic properties; and (4) the stability of residues in hive matrices, which determines the exposure of the colony on the long term. In addition, available information to determine to what extent the current data generated on adult bees are representative of the sensitivity of the species including larvae were reviewed, as it is determinant in identifying the step at which additional data i.e. toxicity data on different growth stages, should be needed.

The possible relevance of an exposure to contaminated honeydew following a soil or seed treatment has been considered. In fact a concentration of a systemic compound that could circulate in the phloem and reach honeydew without harming aphids should in principle not harm a bee foraging on the produced honeydew, unless the compound is highly selective towards non-aphid insects. Selectivity tests should in principle allow highlighting such a selectivity, which would then trigger a specific, tailor-made risk assessment.

Attractiveness of a crop to honey bees

In the context of potential risk to honey bees, the attractiveness of a plant to bees has to be considered according to the possible presence of pollen, nectar (and honeydew) on the crop i.e. a crop can be considered as not attractive to bees when it is harvested before flowering.

A comprehensive list of attractive cropped plants has not been published in the past. Tasei (2001) proposed a list of the crops being visited by honey bees limited to oil seed crops, orchards or vegetable crops, and identified other crops as being only occasionally visited, such as vine or cereals, in case of food shortages.[8] Recent work undertaken in a working group of the AFSSA (French Agency on the Safety of Food) with the aim to provide a guidance document for defining Maximum Residue Limit (MRL) for PPPs in honey has proposed a list of the melliferous plants being attractive to bees based on the presence or not of nectar and honeydew.[9] This list does not include plants such as maize, which may be attractive to bees –and thus be considered in the risk assessment- even if they do not produce nectar. Some recommendations on the factors to consider in assessing the level of attractiveness of a crop are also proposed, such as the presence in the foraging area of other sources of nectar/honeydew of higher/lower level of attractiveness that may influence the behaviour of bees towards the crop of interest. Similarly, the presence of bee-attractive flowering weeds or of "secondary" crops in a non attractive crop may favour visits and lead to some exposure. A description of agricultural practices associated to the crop of concern may help in deciding if visits and exposure are expected or not.

Particular attention should be given to the persistence of residues in soil which may result in an exposure in the case of transfer into rotational crops. The question of the attractiveness is then also raised for crops that enter in the rotation with the treated crop. Criteria to identify persistent substances have been defined in the Directive 91/414/EEC, which in general trigger for additional residue studies involving crop rotation.[1] In the case of residue transfer in rotational crops, investigations to address specifically the risks to bees from attractive plants grown during the rotation with the treated crop become necessary.

Typical residues to be expected in plants, nectar and pollen

Very few published data provide information on the level of residues of PPPs that may be expected in plants or parts of plants following a soil or seed treatment. Some authors could quantify residues in nectar and pollen contaminated by systemic substances being sprayed on crops when blossoms were covered during the spray.[10, 11, 12] Investigations focussing on soil or seed treatment and on residues in nectar or pollen are even more rare. Transfer of substances into honey and royal jelly was proven to be measurable for some systemic substances applied by spray.[13] Evidence of translocation of residues into nectar after soil treatment with granules was demonstrated quite early but investigations based on modern analytical methods are more recent and mainly focus on insecticides[14, 15, 16, 17, 18, 19, 20]

Data on residues of PPPs in plants are systematically generated in the context of the dossiers submitted in support of the evaluation process of PPPs, at least in all cases where the plant is intended to be consumed by humans or animals.[1] A compilation of data generated in various plant species treated with systemic insecticides is presented in Figure 1. This compilation gather residue concentrations measured in all types of plant parts (leaves, fruit, green part, inflorescence, whole plant, and grain) at the period being as close as

possible to blossom, as well as residues measured in nectar and pollen. The results display a majority of samples with less than 1 mg active substance (a .s.)/kg matrix (95th percentile = 0.55 mg/kg, n = 62), the same being observed for degradation products. Taking the matrix nectar and pollen separately, residue concentration would not reach more than 0.1 mg a.s./kg. Compared to the dose applied, the fraction that reaches nectar or pollen may in fact correspond to variable fractions of the dose applied, depending on the plant species and environmental conditions.[15,21] Whether the residue levels measured in whole plants may reflect what is expected to be found in nectar or pollen, very little information could be found in the literature, but the statement that the translocation of pesticides is specified to be measurably less effective to fruiting structures than to other plant parts[22]. This could be related to the role played by flowers hampering as a barrier. Therefore the assimilation of the residues in nectar or pollen to levels equal to those found in whole plants or relevant plant parts at the time of flowering constitutes an assumption protective enough to be considered at a first step. This is consistent with a default transfer factor of 1 considered from the whole plant to the honey in the MRL working group.[9]

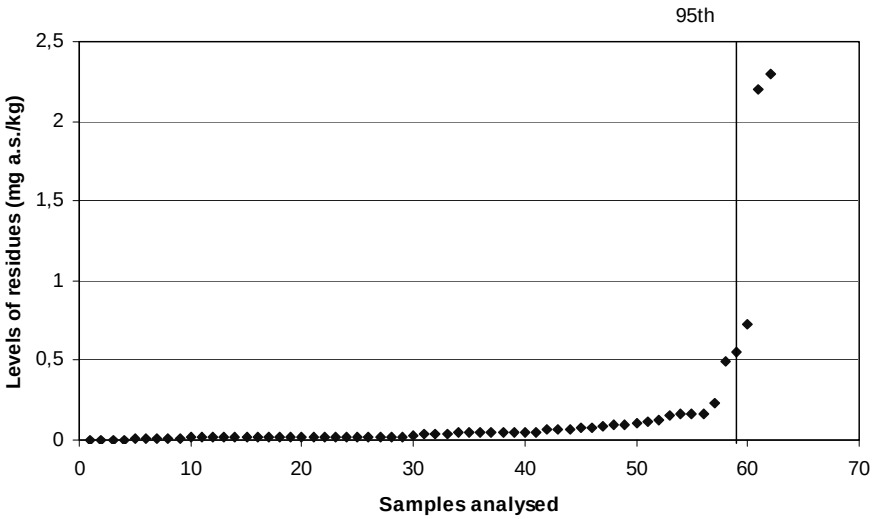

Figure 1 Compilation of residue data in various plant species treated with systemic insecticides. Residue levels, in mg a.s./kg plant, of systemic compounds in diverse types of plant parts (leaves, fruit, green part, inflorescence, whole plant, grain) at the period close to blossoming, as well as residues measured in nectar and pollen (n=62).

Predictability of systemic properties

Attempts to predict systemic properties for PPPs based on chemical and crop factors have been made. From the analysis of phloem translocation of herbicides together with lipophilic properties as the octan-1-ol/water partition coefficient (LogPow) and with dissociation properties as the pKa, the translocation to the plant is expected to be negligible for LogPow values of 4 and above[23] A study on a wider range of substances (ca 400 substances) indicated that the mobility in phloem is satisfyingly predicted by the LogPow, with the pKa modulating the LogPow influence in extreme values[24] Other factors than acidity and polarity may however be implicated into translocation, being related to the substance (such as molecular weight), to the plant (development of the root system, transpiration or nectar production) or to the environment (humidity, light conditions).[15, 22, 23, 24]

In the regulatory context, the information derived from residue studies and plant metabolism studies (residue section of Annex II and Annex III dossiers according to Directive 91/414/EEC), is in general sufficient to identify if the substance will be transferred to the plant during its growth, and if it is further degraded into major degradation products. Similarly, possible uptake of major soil degradation products in plants is identified in these residue studies. This information may then be used to determine whether the substance and/or its residues are to be further considered for a risk assessment to bees. In this respect, the limit of quantification and detection of the analytical methods used in the residue studies must be checked in order to ensure that they were low enough to detect residue levels that exert toxic effects to honey bees. Otherwise additional investigations may have to be considered to demonstrate the absence of translocation at effect levels.

Stability of residues in hive matrices

Again studies that investigate the stability of PPP residues in hive matrices are rather rare. A study on insecticides showed a rather stable behaviour in honey, which may have been related to the absence of Mixed Function Oxydase (MFO) enzymes in the honey sac[25] The residue concentrations in honey, to which larvae are exposed, may also depend on the condensation achievement of honey[26] Therefore it seems premature to consider the variation of residue stability in time in exposure assessments as it may already be done for other organisms exposed to PPPs through the consumption of plant matrices.[3, 27, 28]

Relative sensitivity of larvae compared to adults

Published data that compare the acute toxicity of various pesticides to larvae and adults revealed an important variability.[29] On the 31 substances tested as technical grade or formulated product, three were less toxic to larvae than to adults (the toxicity was considered different when LD_{50} were higher or lower with at least an order of magnitude), 21 were equally toxic and six were more toxic to larvae, no comparison could be made for one substance. No conclusion could be drawn on the predictability of the toxicity to larvae from the chemical family or the mode of action of the tested substance. The highest differences (e.g. ratio between both LD_{50} > 100) were observed for both "simple poisons" (diazinon, profenophos) and Insect Growth Regulators (IGR) (chlorfluazuron). For substances showing a higher toxicity to larvae, the ratio between LD_{50} ranged from 30 (oxamyl) to > 200 000 (chlorfluazuron), the latter being non toxic to adults (LD_{50} > 100 µg/bee, limit test). Differences reached a ratio of 3 to 100. A more recent study that compared the sensitivity of eight substances in adults and larvae, based on the laboratory test of Aupinel *et al.* (2005)[30, 31] indicated that larvae were less sensitive to the assessed compounds than adults with the exception of pirimicarb and metalaxyl[32] In fact, substances acting specifically on growth stages such as Insect Growth Regulators will in many case exert more significant effects when assessed on development parameters than when assessed on adult survival. It may also be true for substances that display, from screening and efficacy studies, and from tests with other non target arthropods, effects specific to juvenile stages. Thus the sensitivity of larvae as well as the related risk assessment should for the time being be considered separately from that of the adults.

Proposed risk assessment scheme

Triggering a risk assessment by establishing exposure

From the review presented above, the relevant parameters that trigger a risk assessment from the exposure to residue of soil/seed treatments are confirmed to be: (1) the attractiveness of plants to bees; (2) a systemic transfer towards pollen and/or nectar and (3) in the case of larvae, a specific risk assessment triggered by the mode of action of the substance of concern, as well as by any observed effect on growth or development as observed on invertebrate species or any other data available in the dossier. The proposed route of entry in a risk assessment scheme takes all these parameters into consideration (Figure 2).

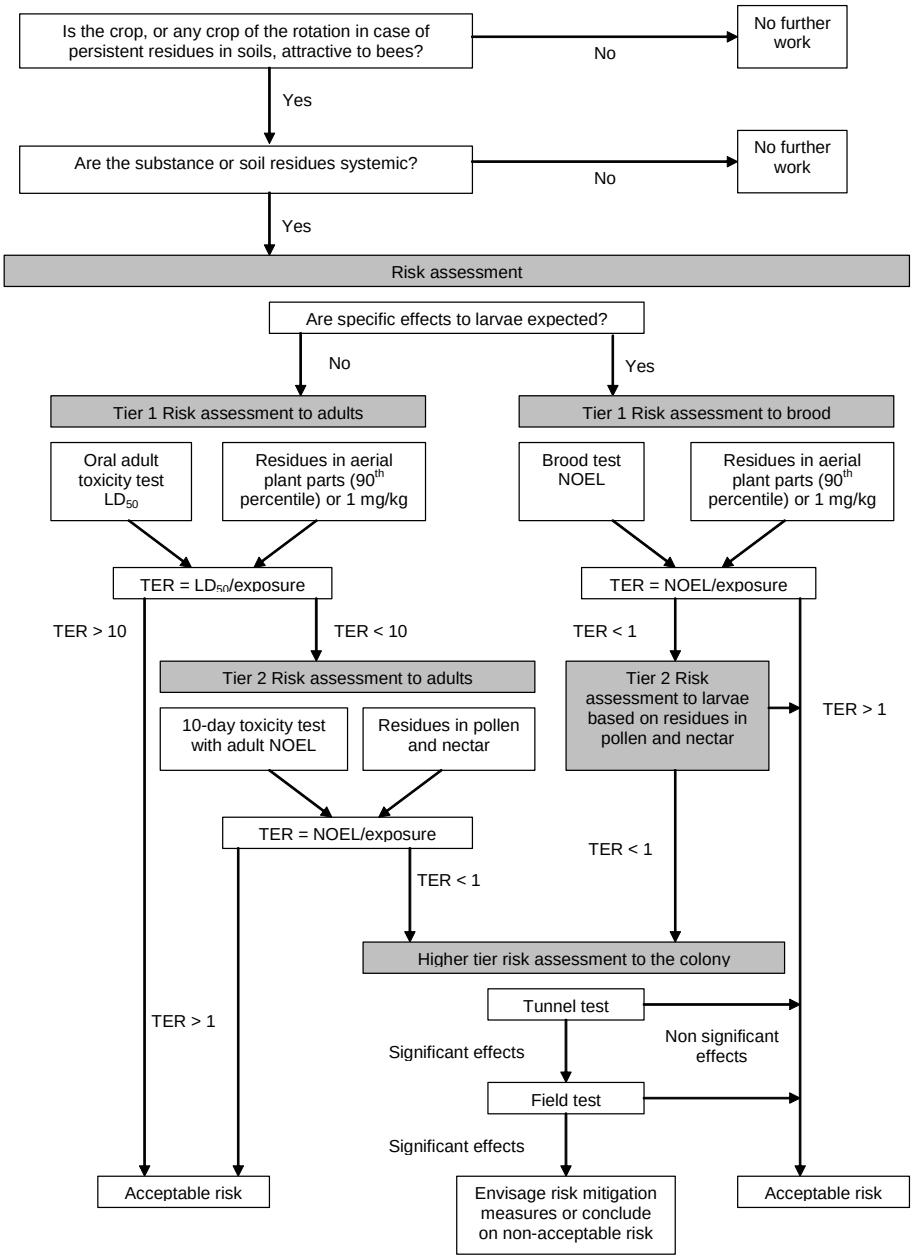

Figure 2 Proposed decision making scheme to evaluate the risks to honey bees in the case of plant protection products applied as a soil/seed treatment. Note that it is possible to skip the Tier 2 and to move directly to a higher-tiered approach.

The attractiveness of the cropped plant to honeybees may be considered as an entry point for this risk assessment. Useful guidance in this respect, as well as recommendations on the criteria to also consider such as the presence in the foraging area of other sources of nectar/honeydew of higher/lower level of attractiveness –which may influence the behaviour of bees towards the crop of interest-, may be found in the document of MRL working group.[9] As stated above, the issue of attractiveness should be considered by also integrating the degradation profile of the substance and its residues in soil, since persistent compounds may also be subject to translocation in plants entering the rotation. In this case, the attractiveness of these plants has also to be taken into account.

At this step, systemic properties trigger the exposure. The prediction of systemic properties may be associated to uncertainties if it is based on chemical properties only. It is therefore proposed to consider all data provided in the residue section of the dossiers submitted in support of authorisation at the national level. A particular attention should be given to the limit of quantification with which they were determined in relation to ecotoxicity thresholds.

It appeared that the sensitivity of larvae cannot be directly extrapolated from that of adults, and exposure of larvae may also be different. The exposure of larvae is triggered by the presence of residues in the hive, which in fact may not be excluded a priori as far as foragers may be in contact with contaminated nectar or pollen at non-lethal levels. The exposure of larvae may however occur through other types of food than for adults. Therefore a separate assessment scheme is proposed for larvae, which should be triggered by the mode of action of the substance of concern as well as by any effect on growth or development observed on invertebrate species, from data available in the dossier.

Risk assessment for adults

In the case that exposure may not be excluded, it becomes essential to assess to what extend this exposure may be of concern. As stated earlier, the level of exposure from the residues actually reaching pollen and/or nectar is rarely available in the current data package. However, the assimilation of the residues in nectar or pollen, to equal levels as found in whole plants or relevant plant parts at the time of flowering (or the generic worst-case value of 1 mg/kg, see 3.3), may provide a protective estimate of exposure levels in a first step.

The assessment of toxic effects may be performed based on current methods that are suitable in this respect.[2,33,34] The main route of exposure of honeybees to soil/seed treatment PPP is probably oral through the consumption of contaminated pollen and nectar, although a contact exposure can not be excluded for bees carrying pollen that contains residues. In this respect the first tier risk assessment could focus on acute oral risks.

The possible risks to bees may as a first tier be quantified through the calculation of a Toxicity Exposure Ratio (TER), as it is currently done for other terrestrial and aquatic organisms.[1] TERs usually correspond to the ratio between a toxicity figure and an exposure levels, expressed in the same units. A TER gives an indication of the margin of safety achieved between the toxicity figure and the exposure level. An acute Toxicity Exposure Ratio (acute TER) may then be calculated based on the acute oral toxicity figure for adult bees and on the assessment of the exposure through estimates of the concentration in the aerial parts of the plant. Because it is an assessment of acute risks, exposure estimates may reflect maximal expected residue levels. The 90th percentile of the data set of residue data for the relevant crop should therefore be selected at this step.

The oral LD_{50} is usually expressed in µg a.s. per bee and residues in plant parts are expressed in mg a.s./kg. Therefore a conversion of residue data is necessary to express exposure as an amount of residue ingested. This conversion may be done by multiplying the 90th percentile of residue concentration (mg a.s./kg plant part) by the daily food ingestion that reflects the dietary need in sugar for a bee. The maximum food ingestion may be estimated from Rortais c. s. at 128 mg /bee/day for nectar foragers.[35] This data set is currently proposed as it is considered to satisfyingly represent food consumption estimates of the different categories of bees. Other figures for food ingestion may become available and could be used if it is demonstrated that they better represent reality.

The calculation of a TER gives an approximation of how close the likely exposure of bees is to a toxicologically significant level. The margin of safety should be sufficient to cover the uncertainty related to longer exposure periods and possible related increased effects. An attempt was made to quantify the range of this uncertainty from existing data. The comparison of toxicity values for adults from acute tests and from chronic (10-day) tests could be done for seven substances.[32] The results showed that the LD_{50} expressed in µg a.s./bee/day as derived from 10-day studies could be derived from 48h LD_{50} by applying an adjustment factor of 10, for acute toxicity data ranging from 0.13 to 90 µg/bee. Despite the need for further work to confirm this correlation with a wider range of compounds, this factor is considered sufficient to cover uncertainties related to the influence of duration of exposure on toxicity levels, considering assumptions regarding exposure levels retained for the tier 1 calculations.

This tier 1 approach is presented in Figure 2. Considering the assumptions that are made to perform this first tier calculation, TER values above 10 are proposed to indicate acceptable risks to bees. In the contrary, TER values below 10 highlight a possible risk to bees and should trigger for a higher tier risk assessment.

Some refinement may be done in a tier 2 approach, by refining the estimate of toxic threshold and/or by refining exposure estimates based on measured level of residues in the relevant material for honeybees.

Additional information with regard to toxic effects may be incorporated by including the duration of exposure of adults in the assessment of effect thresholds. This may be performed by conducting a toxicity test in which worker honeybees are fed with treated sucrose for 10 days in order to calculate a 10-day NOEL (mg a.s./bee/day). The method of Decourtye c.s. allows such an assessment and could be used, despite it is not available as an OECD or EPPO method yet.[36] As stated above, a lower LC_{50} is measured over a 10-day period than after a 4-h ingestion period.[32] Thus uncertainty with regard to chronic exposure to fresh residues is considered to be mostly addressed by the test. If a NOEL derived from 10-day test is used in the TER calculation, the 50th percentile for residue concentration may be used, as it is considered more relevant to reflect a chronic exposure.[3, 27] Revising the toxicity data by generating a 10-day test leads to perform an assessment of the risks for a short-term exposure.

A refinement with regard to exposure may be done by performing measurements of the level of residues in pollen, and for nectariferous plants, in nectar. Measurement should preferably be done in plants grown from coated seeds or sown in a treated soil according to the intended Good Agricultural Practice (GAPs) as the residue levels have to reflect the most probable levels in the crop. Possible build up of residue in soil due to residue persistence, based on Directive 91/414/EEC criteria, and other uses of the substance in the rotation, should be considered if expected. As for plant-based exposure estimates, the mean value of residues levels in pollen or nectar could be used in the TER calculation and compared to 10-day derived toxicity data.

A refinement of both effects and exposure may also be envisaged especially when there is evidence that the refinement of either effect threshold or exposure level will not be sufficient to reach the trigger value. Note however that the trigger value to be used should remain unchanged when a sole exposure refinement is performed since in this case there is still a need to extrapolate from acute to chronic time scale. In the case where a 10-day test is performed, it is proposed to calculate the Tier 2 TER based on the NOEL from the test, as the trigger value to be considered should then be set to 1. Again toxicity and exposure data should be expressed in the same unit. As for the tier 1 calculation, a TER value above the relevant trigger should correspond to acceptable risks, and TER values below the trigger should indicate a possible risk to bees, which should be further investigated through higher tier tests (Figure 2).

Semi-field and field trials

Semi-field and field trials usually correspond to higher tier assessments of the effects a treatment may exert on organisms.[1,2,3] Indeed the aim of higher tier assessment is to address the “unless clause” of the risk assessment which is to “establish through an appropriate risk assessment that under field conditions there are no unacceptable effects on honeybee larvae, honeybee behaviour, or colony survival and development after use of the plant protection product according to the proposed conditions of use”. Thus semi-field and field studies should be designed in order to assess the effects at the scale of the colony, including all bee

categories and long-term effects. Suitable methods to investigate effects of PPPs at this scale are proposed in OEPP/EPPO (2001),[2] which can be adapted to soil/seed treatments. General recommendations for an update of these methods are under development.[37] Nevertheless, specific recommendations to the appreciation of effects of soil/seed treatments are proposed below.

Deciding between a semi-field and a (higher-tiered) field test is a case-by-case decision. Basically, semi-field testing is a suitable option before field testing. The advantage of semi-field tests is that potential mortality is easier to assess and that exposure is ensured and can be easily proven. In semi-field tests, bee colonies are exposed in tunnels to a treated crop. Bees cannot avoid exposure to treated plants, while in field tests, where bee colonies are exposed in plots to the treated crops and are thus free of movements between the crop and surrounding areas.

Semi-field and field trials should be conducted under conditions reasonably representative of the uses to be registered, i.e. using the appropriate crop, application rate and sowing rate. Systemic properties depend on the crop itself, and within a crop the level of exposure is expected to evolve in the same way as the application rate. The duration of flowering should be checked in order to ascertain that it is in the expected range under real cropping conditions. If the substance or its residues are persistent, and if the product may be used on several crops in the rotation, the accumulation in soil should be considered in defining the study protocol.

The effect assessment should consider mortality and foraging behaviour, and effects on bee colonies. In the case pollen or nectar containing residues are brought back to the hive, colonies should be monitored during a sufficient time period to also check long lasting or delayed effects on brood development, queen health, etc.

For both semi-field and field trials, it should be demonstrated that the test bees were exposed under the environmental conditions (especially weather conditions in the case of field trials) of the trial. Parameters such as pollen collection, residue analysis, as well as flight intensity, and observation of the activity on flowers of the treated crop are useful information for that purpose. A quantified assessment of the exposure is particularly important in the case of systemic products, as reference substances for systemic products are difficult to define, being also dependant on crop properties. There should always be a comparable untreated control in order to provide a reference point against which to compare the test treatment. The results, as regards significance of effects should be interpreted with similar rules as for other application modes.[2, 37]

Semi-field and field tests are higher tier data that allow a direct assessment of the effects that may be expected under realistic exposure conditions. Therefore, it is possible to move straight to higher tier investigation instead of refining effect or exposure assessment in a tier 2 approach. This is a case by case decision which should be taken based on the results of the first tier assessment, on the information derived from the properties of the substance and the related expected efforts to propose adequate and easily extrapolable higher tier investigation.

Risk assessment for larvae

A specific risk assessment to bee brood may be necessary when effects on immature stages are unpredictable from the toxicity thresholds observed in adults, e.g. IGRs or other compounds with a specific larvicidal activity (Figure 2). In those cases a stepwise approach similar to that proposed for adults i.e. based on laboratory tests would ideally allow to compare the sensitivity thresholds. Laboratory tests investigating intrinsic properties of the product to immature stages, such as for example the test developed by Aupinel c.s. [30, 31], need further ring testing prior to an implementation as a core data requirement. As regards assessment of effects at the brood scale, a suitable method is described by Oomen *et al.* (1992).[38] Micro colonies are exposed through spiked feeding solution and effects on the brood development are assessed. Ideally, the test should be performed at a level of exposure defined in relation to the mean level of exposure as measured in plant parts, or if available, in nectar or pollen, or other environmentally relevant exposure concentration determined experimentally. The maximum level of exposure supposed to kill foragers should also be considered. Interpretation rules are provided by the authors in the published method.[38] It has to be noted that since exposure level may differ from a crop to another, and considering possible persistence issue in soils,

the results of the test may have to be interpreted in light of the expected level of exposure for each crop of concern. For an adequate risk assessment, the test should allow the determination of a NOEL (No Observed Effect Level) in order to assess the risk for bee brood with e.g. the calculation of a TER that would give an approximation of how closely the likely exposure of bee brood, for a particular crop, is to a toxicologically significant level. Exposure estimates could, as for adults, be deduced either from residues in plant parts (Tier 1) or from residue analysis of nectar or pollen (Tier 2) (Figure 2).

There are too few data available, particularly on exposure of brood, to relate larval toxicity (assessed for example by methods described by several authors, e.g. Wittman & Engels, 1981) with field application rate and brood damage.[39] Therefore, if any effects are detected in a bee brood-feeding test, semi-field or field testing becomes necessary.

It is important to note that as soon as higher tier (semi-field or field tests) tests are triggered, based on the results of lower tier risk assessments for adults or brood, the effects and related risks will have to be addressed at the scale of the colony. This meets the requirements of Directive 91/414/EEC, as stated under annex VI.[1] In this context, potential risks to bees identified from this stepwise approach should be interpreted in light of the uncertainties that remain in the assessment outputs (i.e. variability of exposure levels, ability of the ecotoxicity endpoints) to cover the whole life cycle of the species), and to the measures that may be implemented in the aim to limit or avoid the exposure and thus the risks.

Ability of the scheme to discriminate PPPs AND their use according to risks

In order to verify the ability of the proposed risk assessment scheme to discriminate products that may need a refined assessment for an adequate assessment of the risks to bees from products of low concern, the lower tiers (tier 1 and tier 2) were tested against data available for PPPs.

Acute toxicity studies on adults are performed for all active substances under PPP regulation,[1] so that the risk assessment scheme could be checked for adults. Tier 1 and Tier 2 calculations were performed for all the active substances, existing and new, for which a positive decision with regard to a possible use within Member States has been undertaken at the European level (i.e. included in Annex I of Directive 91/414/EEC). Oral acute LD_{50} were extracted from the French national database *Agritox* as a reference basis for toxicity thresholds.[40] This database is updated with reference data as produced for the re-evaluation of active substances as summarized in the review reports resulting from the European peer review. The database gathered toxicity endpoints expressed as µg a.s./bee, and was built with data generated for technical ingredient exclusively.

Tier 1 TERs were calculated with residue consumption deduced from expected levels in plants. As proposed above, a worst case estimate of 1 mg a.s./kg matrix was considered. This concentration was converted to a daily dose by multiplying this default value (1 mg a.s./kg plant part) by the daily food ingestion reflecting the dietary need in sugar for a nectar foraging bee i.e. 128 mg /bee/day. Resulting Tier 1 TERs were compared to the trigger of 10. Results are presented in table 1.

Table 1 Percentage of active substances that fail the proposed Tier 1 and Tier 2 of the risk assessment (n = 171), which means, from the proposed scheme (see Figure 2): 'Envisage risk mitigation measures or conclude on non-acceptable risk'. Data extracted from the *Agritox* database (http://www.dive.afssa.fr/agritox/index.php)

	Percentage of active substances that do not pass at the first Tiers of the risk assessment (TER < trigger value, n = 171)	**Mode of action**
TER Tier 1 (trigger value: 10)	15.2 %	24 insecticides 1 fungicide 1 nematicide
TER Tier 2 (trigger value: 1)	11.1 %	19 insecticides

The overall discriminating ability of the scheme may be assessed based on an analysis of the Tier 1 calculations. Tier 1 TERs were below the trigger of 10 for 15.2% of the active substances, thus triggering for a Tier 2 risk assessment. The 26 substances in this case consisted mainly of insecticides and acaricides (24 substances), 1 fungicide and 1 nematicide. As insecticides usually display the lowest LD_{50} values, the discriminating ability of the scheme is judged as satisfying. Among the 145 substances for which a Tier 1 TER of 10 or above was calculated, 13 were substances acting as insecticides or acaricides. These substances belong to various chemical families, the most represented being nicotinoids (2) and benzoylureas (2) the latter acting as insect growth regulators. The other substances belong to pyrazolamines, phenoxypyrazoles, triazines, azomethines, tetrazines, benzohydrazides, pyridines, oxazolines or carbamates, and act for example specifically as acaricides. Thus their LD_{50} is found to be higher than 1.8 µg a.s./bee in all cases. In the case of substances that act specifically on developmental stages (proposed as Insect Growth Regulators) or for which an action on juvenile stages has been highlighted from studies on other arthropod species living in terrestrial or aquatic ecosystems, it is in any case recommended to perform a risk assessment focused on larvae (see Figure 2). Note also that within these 13 substances none has been developed to be used as a seed treatment.

Laboratory 10-day toxicity studies are scarcely available for substances or even for PPPs, as the test has not been recommended in the regulatory context. Similarly, residue concentrations in nectar or pollen are available for a very limited number of substances, and related crops. As a consequence, Tier 2 calculations including 10-day test derived NOEC or exposure estimates from nectar or pollen could not be generated. Instead, short-term TERs were calculated considering in a first instance that the results of the study do not indicate stronger effects in the 10-day tests than in acute tests. An arbitrary factor of 3 was considered to extrapolate a NOEL from the LD_{50} in a same test. Of course calculations should in principle rely on a NOEL value as deduced from the study performed. Since a 10-day study is considered to be available, calculations were compared to a trigger value of 1.

A similar approach was followed to check the discriminating ability of the scheme at the Tier 2 level. Tier 2 TERs were below the trigger of 1 for 11.1% of the active substances. The 19 substances in this case consisted mainly of insecticides. This Tier 2 calculation differs from the previous step based on a revised toxicity assessment that supposes no increased toxicity with exposure duration. It is clear that in cases where a 10-fold increased toxicity is observed compared to data from the acute test, this step will become more discriminant than the previous step, and that further refinement of the exposure will become necessary. In this example, a default value was used whereas the scheme recommends the use of the mean residue levels in plant in order to estimate exposure levels. This refinement may also contribute to discriminate substances based on risk assessment criteria.

Conclusions

There was a need for technical guidance addressing the question of the risks to honey bees posed by soil-systemic plant protection product uses under the particular exposure conditions constituted by contaminated pollen or nectar. In developing this guidance, the ICPBR working group considered that the risks posed by plant protection products to the environment have to be dealt with under harmonized conditions within European Member States, and that the risks to non-target organisms should be assessed having in mind a common view about what constitutes an effect at the population level. For these reasons, the proposed scheme meets both EPPO and SANCO guidance documents conception rules, and the approach retained is similar to that for other organisms. A stepwise approach is developed, based on evidence for exposure as an entry route, and that first rely on any existing and relevant data in order to avoid a systematic requirement for field tests. The presented scheme is proposed to update the current EPPO guidance document with a special issue on soil/seed applied PPP, as well as it provides recommendations for conducting higher tier (tunnel and field) studies dealing specifically with soil/seed treatments. Practice is now needed with an emphasis on the higher tier steps, in order to adjust study protocols and conditions for study requirements in the perspective of future amendments of the EPPO scheme.

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Environmental risk assessment scheme for plant protection products - Chapter 10: Honeybees – Proposed scheme

Anne Alix*, Marie P Chauzat, Sophie Duchard, Gavin Lewis, Christian Maus, Mark J Miles, Ed Pilling, Helen M Thompson, Klaus Wallner

*AFSSA-Direction du Végétal et de l'Environnement, 10 rue Pierre Curie, 94704 Maisons Alfort Cedex, France
Phone: 33 1 49 77 21 26, Fax: 33 1 49 77 21 50, E-mail: a.alix@afssa.fr

Specific scope: This standard provides a scheme for assessment of the potential environmental risks presented by systemic plant protection products for honeybees. It is intended as an addition to EPPO standard PP 3/10(2) 'Environmental risk assessment scheme for plant protection products', Chapter 10: Honeybees, revised in 2002-09.

Specific approval and amendment: ICPBR/EPPO working group Honey bees.

Introduction

The sub-scheme in this chapter deals with the potential risks to pollinating insects from the use of soil-systemic plant protection products (PPPs). It specifically addresses the assessment of risks to honeybees (*Apis mellifera*) and their brood and colonies arising from exposure of bees to soil-systemic insecticides and other soil-systemic plant protection products.

As for the assessment of risks arising from sprayed PPPs, it is acknowledged that the most reliable risk assessment is based on data collected under conditions which most resemble normal practice (i.e. by field

tests or by monitoring the product in use). However, beside financial and time costs, these tests pose the question of extrapolating results from one crop to others, since exposure of pollinators is not directly related to the application rate, but also results from systemic properties of the active compound and attractiveness of the crop, which itself is related to agricultural practice. The tiered approach thereafter proposed is thus aiming at triggering higher tier (tunnel and field) testing to the sole cases where an exposure to level of residues of concern can not be excluded. As for other sub-schemes, it is always possible on principle to go straight to higher-tier tests if there is evidence that these tests will be triggered, or for convenience. It should be kept in mind that a multiplication of field tests might be quite heavy since extrapolating exposure conditions between crops can not easily be made.

Risk assessment scheme

Details of the product and its pattern of use

1. Take from Chapter 2 the basic information on the product and its pattern of use.
If this is an insecticide for soil treatment (granules …) or a seed treatment: **go to 2**

Possibility of exposure

2. Is the crop (see note 1) or a rotational crop (see note 2) attractive to bees?
If yes: **go to 3**
If no: **go to 10**

3. Is the active substance or its residues systemic in plants (see Note 3)?
If yes: **go to 4 & 5**
If no: **go to 10**

Preliminary screening based on toxicity and exposure level (Tier 1)

4. Assess the acute toxicity of the active substance to worker honeybees by conducting acute contact and oral laboratory tests. Determine acute LD_{50} for both exposure routes.
Calculate the ratio (TER) between the LD50 (oral) and exposure. Exposure is assessed through the amount of residues that may be ingested by a bee in one day (see Note 4).
If ratio > 10: **go to 11**
If ratio < 10: **go to 7**

5. Does the compound exert sublethal effects on growth or development (risk assessment for bee brood triggered)? (see Note 5).
If yes: **go to 6**
If no: **go to 11**

6. Conduct a bee brood-feeding test with definition of NOEL and TER calculation (see Note 6)
If ratio > 1: **go to 11**
If ratio < 1: **go to 8**

Second tier risk assessment for adults

7. Refine the risk assessment on effects and/or exposure side.

Lethal effects can for example be assessed over a prolonged period that represents the duration of exposure of foragers during flowering (determination of a 10-day NOEL).

Exposure assessment may also be refined by measuring residues in pollen and nectar of the treated crop.

Calculate the new ratio between the NOEL (oral) and exposure. Exposure is assessed through the amount of residues that may be ingested by a bee in one day (see Note 7).
If ratio > 1: **go to 11**
If ratio < 1: **go to 8**

Semi-field trials

8. Conduct a semi-field field trial in conditions representative of use (application rate, crops …) (see Note 8). Are effects on colony survival and development significant (see Note 9)?
If no: **go to 11**
If yes: **go to 9**

Field trials

9. Conduct a field trial in conditions representative of use (application rate, crops …) (see Note 8). Are effects on colony survival and development significant (see Note 9)?
If no: **go to 11**
If yes: **go to 12**

Categories of risk

The preceding stages of assessment allow uses of plant protection products to be allocated to three categories of potential risk to honeybees.

10. Categorize as negligible risk to bees:

11. Categorize as low risk to bees:

12. Categorize as high risk to bees: **go to 13**

13. Review the data which led to the high-risk category and check whether the conclusions are correct (see note 10).
If yes, confirm assessment: **go to 14**
If no, obtain more information as needed: **go to 8**

Risk management

14. The following points give guidance on the steps that might be appropriate in order to mitigate effects on honeybees, for products in each of the categories of risk.

If risk is low (i.e. level of exposure leads to acceptable risks) or negligible (i.e. no exposure): set no restrictions on use.

If there is a high risk consider conditions that would limit or exclude exposure of bees. For example, allow use only in crops which are not visited by bees. Consider the persistence of residues in soil and possible exposure through rotational crop and consider related recommendation with regard to rotational crops in contaminated soils. Mitigation measures should be proposed as moving beehives away from the treated crops.

According to the Directive 2003/82/EC, these indications or restrictions should be mentioned in standard phrases for safety precautions for the environment as SPe8: “Dangerous to bees/To protect bees and pollinating insects …”. Specific phrases may be proposed based on the conditions that would lead to a limited or excluded exposure of bees.

Explanatory notes

Note 1 Establish if the crop is attractive to bees

The attractiveness of the cropped plant to honeybees may be considered as an entry point for this risk assessment. Useful guidance in this respect, as well as recommendations on the criteria to also consider such as the presence in the foraging area of other sources of nectar/honeydew of higher/lower level of attractiveness, i.e. weeds, which may influence the behaviour of bees towards the crop of interest, may be found in the document of MRL working group (EC, 2009). In general, a crop can be considered as not attractive to bees when it is harvested before flowering. Some plants being not intrinsically attractive to bees may be visited due to extra floral nectarines, e.g. in field beans or due to honeydew produced by aphids on

crops otherwise not attractive to bees. Similarly, the presence of bee-attractive flowering weeds or of "secondary" crops in a non attractive crop may favour visits and lead to some exposure. A description of agricultural practices associated to the crop of concern may help in deciding if visits and exposure are to be expected or not.

Note 2 Establish if rotational crops have to be considered in the risk assessment

The persistence of the product in soil may result in an exposure of bees, in the case of the growth of an attractive plant in the rotation. Criteria to identify persistent substances have been defined in Directive 91/414/EC, which in general require additional residue studies involving crop rotation. In the case of residue transfer into rotational crops, investigations to address specifically the risks to bees from attractive plants grown during the rotation with the treated crop become necessary.

Note 3 Establish if the substance or its residues present systemic properties

The exposure of honeybees to plant protection products used for soil or seed treatments may occur in the case of transfer of the active substance itself or its degradation products to the parts of the plant that may be consumed by bees, i.e. nectar or pollen, or honeydew. Exposure to contaminated honeydew is, however, not considered a relevant route in the case of soil and seed treatments, as (a concentration of) a systemic compound that could circulate in the phloem and reach honeydew without harming aphids should in principle not be capable of harming a bee foraging on the produced honeydew, unless the compound is highly selective towards non-aphid insects. Selectivity information (as apparent from the registration dossier) should in principle allow highlighting such a selectivity, which would then trigger for a dedicated risk assessment according to the present sub-scheme.

Information derived from residue studies and plant metabolism studies (residue section of Annex II and Annex III dossiers according to Directive 91/414/EC), is in general sufficient to identify if the substance is transferred into the plant during its growth, and if it is further degraded into major degradation products. Similarly, possible uptake in plants of major soil degradation products is identified in these residue studies. In case of uptake and transfer into the plant, the PPP is systemic, and the answer to question 3 is 'yes'.

The sensitivity (i.e. limit of quantification and detection) of the analytical methods that were used in the residue studies must be checked in order to ensure that they were low enough to detect residue levels that exert toxic effects to honey bees. If uncertain that detection methods were sensitive enough, additional investigations have to be considered to demonstrate the absence of residue translocation at toxic levels. Beside this verification, studies that specifically investigate the presence of residues in flowers, nectar or pollen are not necessary at this stage.

Note 4 First tier risk assessment

Suitable methods for acute oral and contact toxicity tests are described by OEPP/EPPO 170 (2001), OECD (1998a, b).

The main route of exposure of honeybees to soil/seed treatment is oral through the consumption of contaminated pollen and nectar, although a contact exposure can not be excluded for bees carrying pollen that contain residues. It has to be noted, however, that topical exposure through contaminated nectar may also occur for sprayed, non-systemic compounds.

In this respect the first tier risk assessment focuses on acute oral risks. A first tier toxicity exposure ratio (TER) is calculated based on the acute oral toxicity figure for adult bees and on an assessment of the exposure through, ideally, pollen and nectar. Residues in pollen and nectar are rarely quantified in residue studies that are available in the residue section of dossiers as these studies are performed for other (risk to consumers) purposes. The transfer and fate of products and their residues in plants is not homogeneous, and transfers to the blossom depend on their ability to cross the flower barrier. Thus estimates of the concentration in the aerial parts of the plant may be considered as an overestimation of residual concentration in nectar and pollen, and thus provide a useful margin of safety as a first assessment step. In the case such data on residues in plant material are not considered reliable or available, a generic worst case

value of 1 mg (a.s.)/kg plant matrix is proposed. This value is deduced from a compilation of the data generated in various plant species treated with systemic insecticides and the consequent residue concentrations measured in all types of plant parts (leaves, fruit, green part, inflorescence, whole plant, and grain) at the period being as close as possible to blossom, as well as residues measured in nectar and pollen. The results displayed a majority of samples with less than 1 mg active substance (a.s.)/kg matrix (95th percentile = 0.55 mg/kg, n =62), the same being observed for degradation products. Taking the matrices nectar and pollen separately, residue concentrations would not reach more than 0.1 mg a.s./kg.

Because it is a worst case assessment, exposure estimates should reflect the maximal expected residue levels. When based on measured residue in plant matrices, the 90th percentile of the data set of residue data for the relevant crop should be selected at this step.

The oral LD_{50} is measured in µg active substance per bee and residues in plant parts are expressed in mg/kg. Therefore a conversion of residue data is necessary to express exposure as an amount of residue ingested. This conversion may be done by multiplying the 90th percentile of residue concentration (mg a.s./kg plant part) by the daily food ingestion that reflects the dietary need in sugar for a bee. The maximum food ingestion may be estimated from Rortais et al., 2005 at 128 mg /bee/day for nectar foragers. The data set provided by Rortais et al. (2005) is proposed as it is considered to satisfyingly represent food consumption estimates of the different categories of bees. Other figures for food ingestion may become available and could be used if it is demonstrated that they better represent reality.

The calculation of a TER gives an approximation of how closely the likely exposure of bees is to a toxicologically significant level. The margin of safety achieved should be sufficient to cover the uncertainty related to longer exposure periods and possible related increased effects. To quantify the range of this uncertainty, the comparison of toxicity values for adults from acute tests and from chronic (10-day) tests was done for 7 substances (Defra, 2007). The results show that the LD_{50} expressed in µg a.s./bee/day as derived from 10-day studies can be derived from 48h LD_{50} by applying an adjustment factor of 10, for acute toxicity data ranging from 0.13 to 90µg/bee. Despite the need for further work to confirm this correlation with a wider range of compounds, this factor is considered sufficient to cover uncertainties related to the influence of exposure duration on toxicity levels.

Note that for low toxicity figures (e.g. LD_{50} of 10 µg a.s./bee and above, TER calculations will always result in values above the trigger (= low risk) even with exposure levels estimated from concentrations in aerial parts. However, a definite cut-off value for entering in the risk assessment scheme through a Tier 1 TER is difficult to establish. As the Tier 1 calculation does not involve additional experiments but the acute oral toxicity test in adults, some toxicity-based trigger is not deemed necessary.

Note 5 IGR

Insect growth regulators (IGRs) and substances that display effects specifically to juvenile stages, apparent from screening and efficacy studies and from tests with other non target arthropods (including terrestrial and aquatic), have to be assessed more precisely with a bee brood-feeding test (Note 6).

Note 6 Bee brood-feeding tests

A suitable method is described by Oomen et al. (1992). The test should be performed at the highest expected level of exposure (the maximum level of exposure is supposed to kill foragers) as measured in plant parts, or if available, in nectar or pollen, or other environmentally relevant exposure concentration determined experimentally.

As the level of exposure will vary from a crop to another and probably also between samples of a same crop, it is not necessary to duplicate the study to take the variability of exposure levels into account. Rather, the test should allow the determination of a NOEL (No Observed Effect Level) in order to assess the risk for bee brood with e.g. the calculation of a TER that would give an approximation of how closely the likely exposure of bee brood, for a particular crop, is to a toxicologically significant level. Note that since exposure level may differ from a crop to another, and considering possible persistence issues in soils, TER calculations should be done for each crop separately, to ensure that the trigger is reached in any case.

There are too little data available, particularly on exposure of brood, to relate larval toxicity (assessed for example by methods described by several authors, e.g. Wittman & Engels 1981) with field application rates and brood damage. Therefore, if any effects are detected in a bee brood-feeding test, semi-field or field testing becomes necessary.

Note 7 Second tier risk assessment

Additional information with regard to toxic effects may be incorporated by including the duration of exposure of foragers in the assessment of effect. This should be performed by conducting a toxicity test in which worker honeybees are fed treated sucrose for 10 days to calculate a 10-day NOEL (mg a.s./bee/day). The method of Decourtye et al. (2005) could be used, although it is not available as an OECD or EPPO method yet. Usually a lower LC_{50} is measured over a 10-day period than after a several hours ingestion period (Defra 2007). Thus uncertainty with regard to chronic exposure to fresh residues is considered to be addressed by the test.

A refinement of the exposure may be made by measurements of the residues in pollen, and if relevant, nectar, in plants grown from coated seeds or sown in a treated soil according to the intended Good Agricultural Practices (GAPs). Residue levels have to reflect the levels expected from the crop. Possible build up in soil due to residue persistence, based on Directive 91/414/EC criteria, and use of the substance in the rotation should be considered if expected. Since exposure has to reflect a period of several days, the mean value of the concentrations measured in samples could be used in the TER calculation.

The tier 2 TER should be calculated, with the NOEL from the 10-day chronic toxicity test in bees and/or the measured level of residues in the relevant material for honeybees (mean residue data). A further refinement of both effects and exposure is not necessary but it is rather to be considered as a possibility, especially when there is evidence that the refinement of either effect threshold or exposure level will be sufficient to reach the trigger value. If a 10-day test derived NOEL is used in the TER calculation, the 50th percentile for residue concentration may be used, as it is considered more relevant to reflect a chronic exposure. Note however that the trigger value remains unchanged in the case of a single exposure refinement since the uncertainty with regard to chronic effects remains. Again toxicity and exposure data should be expressed in the same unit.

Note 8 Semi-field and field trials

Suitable methods for semi-field and field trials are discussed in OEPP/EPPO (2001) and can be adapted to soil/seed treatments.

Semi-field and field trials should be conducted under conditions reasonably representative of the uses to be prescribed (appropriate application and sowing rate and crop). This allows also for testing under specific conditions of exposure (e.g. in relation to duration of flowering) to be expected. If the substance or its residues are persistent and the product may be used on several crops in the rotation, the accumulation in soil should be considered the study protocol.

Possible effects on adult survival and foraging behaviour and on bee colonies should be checked. In case pollen or nectar containing residues are brought back to the hive, colonies should be monitored during a sufficient time period to also check long lasting or delayed effects.

For both semi-field and field trials, it should be demonstrated that the test bees were exposed under the environmental conditions (especially weather conditions in case of field trials) of the trial. Parameters such as pollen collection, residue analysis, as well as flight intensity, and observation of the activity on flowers are useful information for that purpose. A quantified assessment of the exposure is particularly important for systemic products, as reference substances for systemic products are difficult to define, being also dependant on crop properties. There should always be a comparable untreated control in order to provide a reference point against which to compare the test treatment(s).

Semi-field testing is a suitable option before field testing. The advantage is that potential mortality is easier to assess and that exposure is ensured and can be easily proven.

Semi-field testing is readily feasible by exposing bees to a treated crop in tunnels. This reflects a truly realistic scenario in so far as that there can not be a certain target exposure level accurately pre-determined, since a certain dressing rate of seeds will not necessarily lead to an exactly predictable residue level in nectar and pollen. Of course this is also a characteristic of natural conditions that a certain level variation can occur. An alternative for special design tunnel studies, where exposure of the bees to a certain pre-determined residue level is aimed at, could be the exposure to spiked nectar and pollen in a tunnel, as far as technically feasible. The choice between these options should then be made on a case-by-case basis according to the particular circumstances of the situation.

Note 9 Significance of semi field/field results

Effects as a result of the experimental treatment in semi-field or field trials may be difficult to assess and to distinguish from other sources of mortality. Statistical analysis of the results normally solves this problem and studies should be designed to allow statistical treatment to be performed.

Current procedures, including pollen collection, possible residue analysis of collected pollen and direct observations of foraging behaviour should provide sufficient information concerning exposure to the test compound to enable reliable interpretation of results. Although exposure a priori is ensured in cage or tunnel trials, analysis of nectar, pollen or blossoms is recommended in order to verify the residue level in bee-relevant matrices.

Note 10 Additional investigation

Special effects (larval toxicity, long residual effect, disorienting effects on bees, etc.) identified by the field test may in some cases require further investigation using specific methods, particularly in the case these effects are observed under realistic exposure conditions, since this means they may be expected also under the intended conditions for use of the PPP. Such investigations should then be dedicated to appreciate the importance and significance of effects and to help in setting risk mitigation measures.

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Proposed revision of the higher tier testing requirements for EPPO Standard PP1/170: Test methods for evaluating the side-effects of plant protection products on honeybees

Gavin Lewis*, Mike Coulson, Christine Vergnet, Christian Maus, Helen M Thompson, Roland Becker, Dick Rogers, Ingo Tornier

*JSC International Ltd., Simpson House, Windsor Court, Clarence Drive, Harrogate HG1 2PE, UK
Phone:+44(=)1423 520245, Fax: +44(0)1423 520297, E-mail address:Gavin.Lewis@jsci.co.uk

Abstract

Background: Regulatory evaluations for the effects of pesticides on honeybees in the EU are based on the honeybee test guidelines and risk assessment scheme of the European Plant Protection Organisation (EPPO). While this is considered to be robust and effective, it is also recognised that a continuous process of review and appropriate development is necessary. A working group of the International Commission for Plant-Bee Relationships (ICPBR) had been set up to review the current guidance set out in the EPPO PP1/170 standard for higher tier testing i.e. semi-field (cage) test and full field studies. The aim of this group was to utilise the considerable experience obtained with honey bee testing. This paper presents the working group's proposed revision to the EPPO standard PP1/170, taking into account feedback received from the 10th ICPBR Symposium in Bucharest.

Results: The primary aim of the group has been to produce guidance that is sufficiently detailed yet suitably flexible so that it enables tests to be conducted and evaluated without being too prescriptive. In particular, it recognises that higher tier testing may arise as a result of various initial concerns e.g. adult toxicity, brood effects and systemic toxicity. The guidance is designed to provide the different emphasis that is required to meet the specific requirements of individual studies.

Conclusion: The revision of higher tier testing for honeybees presented in this paper is proposed as an update to the current EPPO PP1/170 standard.

Keywords: honey bees, test guidelines, higher tier, semi-field (cage) tests, field tests.

Introduction

Currently in the EU, regulatory evaluations for the effects of pesticides on honey bees are based on the honey bee test guidelines and risk assessment scheme of the European Plant Protection Organisation (EPPO)[1]. The International Commission for Plant-Bee Relationships (ICPBR) Bee Protection Group provides the technical input to the EPPO standard PP1/170[2] and associated risk assessment scheme[3]. While the current EU risk assessment scheme is considered to be robust and effective it is also recognised that a continuous process of review and appropriate development is necessary. This needs to be done in a considered way with the development of a consensus view amongst the expert representatives within the group. This allows any new information to be evaluated and its significance in relation to the risk for honey bees assessed. Accordingly, a review was carried out in 1999 at the 7th ICPBR symposium in Avignon, France[4] and this resulted in the current versions of the EPPO standard PP1/170[2] and the associated risk assessment scheme[3]. More recently, EPPO had asked the ICPBR Bee Protection Group to undertake a similar exercise at the 10th Symposium in Bucharest.

At the previous meeting of the Bee Protection Group (in 2005 at the Central Science Laboratory, York, UK)[5], a working group was set up to review the current guidance for higher tier testing i.e. semi-field (cage) test and full field studies. The aim of the EPPO standard PP1/170 is to provide sufficient guidance to allow the studies to be conducted without being too prescriptive. It was considered that this should be looked at again in the light of experience obtained with the working of this guideline over many years. In particular, it was recognised that developments in other areas highlighted the fact that higher tier testing might be triggered via a number of different routes e.g. adult toxicity, brood effects, systemic activity etc.

Accordingly, it is important that the guidance is sufficiently detailed and flexible to address the different emphasis that each requires.

This paper presents the working group's proposed revision to the higher tier testing requirements of the EPPO standard PP1/170, revised in response to comments received at the 10th ICPBR Symposium in Bucharest and after circulation to all delegates following the meeting.

Semi-field tests

Semi-field testing (cage, tunnel or tent tests) are higher tier studies that may be triggered as a result of the standard Tier 1 risk assessment i.e. contact or oral hazard quotients >50. In addition, it may be triggered as a result of possible concerns about systemic activity identified during the Tier 1 assessment or by information about insect growth regulator (IGR) properties. Semi-field testing can also be modified for specific assessments with honeybees e.g. repellency and other behavioural effects, effects of aged residues, the evaluation of the hazard of the application of plant protection products to honeybees foraging the honeydew secreted by aphids or for specific testing of brood effects. It is therefore important that this guideline is interpreted with appropriate flexibility to ensure that all these requirements can be accommodated. Similarly, it is important when designing a semi-field study that the aims and objectives are clearly specified.

Experimental conditions

Principle of the trial

Honey bees from small colonies are forced to forage on a flowering crop in field cages (to provide realistic worst-case exposure). Typically, the test products and a toxic standard known to present a high hazard to bees (e.g. dimethoate) are applied in separate cages during bee flight, while other cages are left as untreated or water-sprayed controls. The toxic standard is used to confirm that the bees are exposed to the treatment and to calibrate the magnitude of the possible effects under trial conditions. Its selection should be based on the specific concerns being addressed. In those cases where the trial conditions do not allow the use of a toxic standard (e.g. in the case of assessment of systemic activity), this needs to be justified and it should be demonstrated otherwise that bees have been exposed. The effects of the treatment on bees are assessed just before and several times after application.

Trial conditions

As a guide, cages should contain a minimal crop area of 40 m^2. However, cages of a smaller or significantly larger size may be appropriate depending on the objectives of the study. A number of factors need to be considered when selecting the appropriate cage size e.g. nature and attractiveness of the test crop, objectives of the study (short versus longer term effects) and the size of the test colonies. For screening purposes and the study of specific questions such as short term mortality assessments on aged residues, smaller cages (of at least 12 m^2) may be appropriate. For increased realism or where increased foraging area is required, larger cages may be appropriate. The cage should have a mesh size that the bees cannot escape through e.g. ≤3 mm.

In the first instance, rape, mustard, *Phacelia* or another crop highly attractive to bees should be used as test plants e.g. in the case of a standard semi-field trial based on acute toxicity. In other cases, identification of a surrogate (worst-case) test crop may be more difficult e.g. for systemic compounds, where the test crop should be one for intended use. Other factors may then need to be considered when extrapolating between crops (e.g. plant metabolism data). Less attractive crops (on which use of the product is proposed) may be appropriate e.g. if significant effects are seen or expected with the standard attractive crops. This will have implications for the design and conduct of the study, e.g. a toxic standard may not be appropriate and the levels of foraging expected will be lower. Normally, treatments should be applied when the test crop is in full flower except where justified e.g. when recommended product use is pre-flowering.

On cereals, where aphid honeydew is being simulated, sucrose solution is sprayed onto a suitable crop e.g. wheat, in such a manner as to maintain sufficient attraction. Such testing may require larger areas of crop to provide sufficient forage for the test colonies and thus may require the use of a larger cage. For such a test, trial conditions and methods described by Shires *et al.* (1984)[6] are suitable.

Preparation of the bees

Use one small healthy queen-right colony per cage containing approximately 3,000 to 5,000 bees and at least three full frames containing all brood stages and stores of nectar/pollen (but not excessive in order to ensure exposure to the treatments), or a nucleus. The size of the colony may need to be adjusted according to the aims and conditions of the study. Thus, normal field colonies may be used in larger cages while in small cages only one brood frame and one frame with nectar/pollen may be sufficient. For the assessment of brood effects, smaller colonies may also be appropriate e.g. 'Mini-Plus-Beuten' hives, according to the method of OECD Guidance Document 75[7]. Feeding of the colonies during the trial may be necessary depending on the available forage and water should be offered.

Design of the trial

Treatments: test product(s), toxic standard known to present a high hazard to bees (e.g. dimethoate for a standard assessment based on acute toxicity) and a control without plant protection product. The choice of toxic standard will depend on the objectives of the study (e.g. fenoxycarb for an IGR compound) and may not be appropriate in some cases (e.g. for systemic compounds). The control should normally receive a water spray unless there is a justified reason for not doing this.

Test units: cages with one colony each.

Replicates: sufficient to allow appropriate risk assessment. 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) or where a high number of treatment groups are required. Where this is the case, smaller cages may allow replicate numbers to be maintained although this needs to be considered in the context of the study objectives and the nature of the information required.

Application of treatments

Test Product(s): use formulated products only.

Timing of application

Normally the products should be applied during the daytime when bees are foraging most actively. However, this may be modified if appropriate for the objectives of the study e.g. when testing systemic compounds applied pre-flowering (seed dressings and soil applied products) or for assessing mitigation measures (application before bees are active). To assess aged residues, application is carried out at intervals before exposure, which can take place in the same way as for directly sprayed treatments. Untreated pot-grown plants in the cages are then replaced with the treated ones after appropriate ageing intervals. There should not be any rainfall before directly sprayed applications have dried e.g. for about 2 hours after application.

Shortly before application the number of foraging bees per m^2, and how the assessments are carried out, should be recorded. Where a toxic standard has not been used, a foraging density of at least 5 bees/m^2 is required on bee attractive crops (e.g. *Phacelia*) in order to verify exposure. However, in other cases foraging levels need to be related to the specific conditions of the trial e.g. for less attractive crops and pre-flowering application of systemic compounds (where exposure is related to a more sustained period during flowering).

Application rates

The product should normally be applied at the highest rate specified for the intended use in flowering crops. Lower application rates may be applied e.g. if the off-crop risk needs to be assessed (using drift rates of application), when exposure on weeds in orchards are tested (ground deposition rates), or in cases where

products which are intended for use in three-dimensional crops and where the use rate is dependent on the canopy height (but the test is being conducted in a 'two-dimensional' surrogate crop). Normally a single application will be sufficient but multiple applications (according to the GAP) may be appropriate in specific cases e.g. for sprayed compounds that have the potential to move to the flowers via foliar uptake.

Mode of assessment

Pre-treatment assessments should be sufficient to demonstrate stable background mortality and to show that the bees have acclimatised to the test conditions and are actively foraging on the crop. Typically, for a standard study with a sprayed product this means that the colonies need to be introduced into the cages approximately 2-3 days prior to treatment. This will not be possible where a pre-flowering treatment is being tested. In this case, the hives are introduced at flowering and exposure starts straight away. In the case of aged residues, exposure can take place by replacing untreated pot-grown plants used to acclimatise the bees with plants previously treated at appropriate intervals.

Conduct mortality and behavioural assessments at least 2 days prior to treatment (to demonstrate the bees are acclimatised) and then just before and at several intervals after treatment (preferably daily but at least on days 0, 1, 2, 3, 5 and 7). Additional assessments can be carried out if appropriate e.g. on treatment day. Longer post-treatment periods may be required in some cases but will be limited by the confinement of the colonies (subject to specific test conditions). Normally 7 days is the appropriate post-treatment exposure period, which will be limited by the flowering period of the crop or the confinement of the bees to a limited foraging area. Record flight and/or foraging activity in the cages as given by the number of bees/m^2 (y monitoring a fixed area e.g. 1 m^2, or using transects along the length of sub-plots (if present), in both cases for a defined period. The details of these assessments will depend on a number of factors e.g. cage size and attractiveness of the crop, but they should be sufficiently reliable to quantify the activity level. The behaviour of the bees on the crop and around the hive should be recorded using a standardised approach. Count the dead bees in dead-bee traps and those dying in the rest of the cage (e.g. from water permeable sheets placed along paths or around the edge of the crop).

The condition of the test colonies (including brood status) should be assessed once just before exposure (e.g. when moving the colonies into the cages) and once at the end of exposure. However, due to their confinement post-treatment assessments are of limited use unless the trial has been specifically designed to address this (e.g. OECD guidance document 75 [7]). Other assessments should be made as appropriate to the type of test product and the test design. As the colonies are confined and their foraging activity is greatly restricted, additional endpoints that are sometimes included in longer-term, full field trials e.g. pollen and nectar storage and hive weight development, are generally not appropriate for cage tests. If such restrictions represent a significant limitation in the context of the study objectives it may be necessary to go straight to a field trial (an option always available within the context of the risk assessment scheme). Residue analysis may be appropriate in specific cases to verify exposure e.g. systemic compounds. Record temperature, humidity, rainfall and cloud cover at appropriate intervals throughout the assessment period (in the cages where appropriate). Alternatively, use data from the nearest official weather station.

If it is appropriate to follow the colonies for longer periods (e.g. to assess colony development or to consider the possibility of delayed effects or delayed exposure from stored pollen/nectar) they will need to be moved into the open at another site. The 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 flowering crops present), in order that they are not exposed to different location-specific factors. The collection of untreated pollen and nectar from non-crop plants by the test colonies at this stage cannot be avoided and reflects normal field conditions.

Results

Repeat tests where control mortality is excessively high and also where effects in the toxic standard treatment are low*****(see Footnote p. 41). While there should be a statistically significant increase in effects with the toxic standard compared to the untreated control (as appropriate to the mode of action of the compound) the actual level will depend on the trial conditions (e.g. the attractiveness of the test crop) and so it is not always appropriate to set a required level.

Mortality, behavioural and colony assessment data must always be provided and any other data which is relevant to the properties of the product being tested. Adjustments may be needed for differences between colonies in pre-treatment levels of some parameters e.g. mortality and foraging levels.

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 recognised that there are endpoints which are intrinsically suitable for statistical evaluation (e.g. mortality data) whereas others may be not (e.g. behavioural endpoints). In addition, the evaluation needs to consider the range of parameters assessed and their relative importance, which will depend on the specific objectives and design of each study and must be considered on a case-by-case basis. The evaluation of the results also needs to take into account the biological significance of any effects seen in the context of each colony and the test conditions and this will involve some degree of expert judgement.

Field tests

As for semi-field studies, field testing may be required as a result of a number of possible reasons e.g. the Tier 1 risk assessment based on hazard quotients, systemic activity, concerns about potential brood effects or based on the results of cage studies. Again, it is important that this guideline is interpreted with appropriate flexibility to ensure that the specific requirements are addressed and that the aims and objectives of each field study are clearly specified

Experimental conditions

Principle of the trial

Honey bee colonies should be placed in or on the edge of large test fields of flowering crops. The fields should be chosen so that bees are mainly exposed to the flowering field in which the hives are placed. Test fields should be well separated to minimise bees foraging on neighbouring treatments. The treatments are applied to separate test fields, normally during the daytime when bees are foraging most actively. However, this may be modified if appropriate for the objectives of the study e.g. when testing systemic compounds applied pre-flowering or for assessing mitigation measures.

A toxic standard is usually not suitable for field trials. In specific cases a toxic standard known to present a high hazard to bees may be used. In those cases where a toxic standard is not included, it should be demonstrated otherwise that bees have been exposed. Reference products that present known hazards to bees may also be included for comparison with the test product. Assessments are made to assess possible effects on the bees shortly before and several times after application.

As with the semi-field tests, it is intended that this guideline should be interpreted with appropriate flexibility to accommodate differing requirements arising from initial (lower tier) assessments. The aims and objectives should be clearly identified to reflect this.

Selection of the crop

In the first instance, rape, mustard, *Phacelia* or another crop highly attractive to bees should be used as test plants in the case of a standard field trial based on acute toxicity. In other cases, identification of a surrogate (worst-case) test crop may be more difficult e.g. for systemic compounds, where the test crop should be one

for intended use. Other factors may then need to be considered when extrapolating between crops (e.g. plant metabolism data). Less attractive crops (on which use of the product is proposed) may be appropriate e.g. if significant effects are seen or expected with the standard attractive crops. This will have implications for the design and conduct of the study e.g. a toxic standard may not be appropriate and the levels of foraging expected will be lower. Normally, treatments should be applied when the test crop is in full flower except where justified e.g. when recommended product use is pre-flowering.

Trial conditions

Place the colonies in or on the edge of the flowering crop on which exposure will take place. In the case of applications during flowering, the colonies are placed in position approximately 2-3 days before the trial to ensure that bees are foraging mainly in the test plot on the day of treatment, as bees tend to begin foraging in areas immediately adjacent to their hives. The trial schedule should take into account the flowering (exposure) period of the specific test crop being used. In other cases, the timing for the placement of the colonies will depend on the specific trial objectives e.g. at the start of exposure in the case of systemic compounds. During spray applications, the test hives should be protected from spray drift.

Preparation of the bees

Use healthy, well-fed, queen-right colonies in normal condition that contain at least 10,000 to 15,000 bees, according to the season. Each colony should cover at least 10-12 frames, including at least 5-6 brood frames (nectar/pollen stores should not be excessive, especially where brood effects are a specific objective of the study). If colonies differ in size, ensure equitable distribution between treatments. Specific colony size and set-up may be adapted according to local beekeeping practice.

Design and lay-out of the trial

Treatments: product(s) to be tested and an untreated control; reference product(s) that present a known hazard to bees may be included, for comparison. As a toxic standard is normally not included, honeybee exposure should be otherwise demonstrated e.g. by evidence based on assessments of foraging bees before and after application (collecting pollen and marking bees in the field or at the hive may also provide useful information in this respect).

Plot size: The area of each plot required will depend on a number of factors e.g. the number and size of colonies, the crop type and seasonal timing, but should be large enough to provide sufficient forage to ensure appropriate exposure of the test bees. In the case of the standard attractive crops, 2500 to 5000 m^2 for *Phacelia* and approximately 1 ha for rape and mustard are appropriate. This should be considered in relation to the total number of bees (proportion of the foraging population) exposed. In the case of *Phacelia*, plots may need to be irrigated to ensure that the crop remains sufficiently attractive. Plots should be well separated to avoid bees foraging on the wrong plot (2-3 km depending on local conditions) but should be as homogenous (e.g. microclimate, exposure and surrounding landscape) as reasonably practicable. The distance between plots should be recorded. The plots should not be close to other flowering crops or non-cultivated areas which are significantly attractive to bees. As a guide the same separation distance as for the test plots should be considered, taking into account the size and attractiveness of the other crops or non-cultivated areas. Bee attractive weeds in the vicinity of the test plots cannot be avoided but it may be useful to record them during the exposure phase when considered significantly abundant.

Replicates: although very desirable, replication is often not feasible because of the requirements for separation.

Number of colonies per treatment/plot: Use at least 4 colonies per treatment (related to plot size and attractiveness of crop). Additional colonies may be needed for specific purposes e.g. for pollen traps. No large apiaries should be present in the area around the trial plots and if bee colonies other than those used in the study are present in the immediate vicinity, they should be recorded.

Application of treatments

Test Product(s): use formulated products only.

Toxic standard/Reference product(s)

A toxic standard is usually not suitable for field trials. In specific cases a toxic standard known to present a high hazard to bees may be used. In those cases where a toxic standard is not included, it should be demonstrated otherwise that bees have been exposed. Reference product(s) that present known hazards to bees may also be included for comparison with the test product.

Timing of application

Application timing should depend on the study objectives. Thus, for a standard field trial based on acute toxicity, the treatments should be applied during the daytime when bees are demonstrated to be actively foraging on the test crop. This may be modified e.g. when testing systemic compounds applied pre-flowering (seed dressings and soil applied products) or for assessing mitigation measures. Treatments should be applied in as short a time period as technically feasible, ensuring that conditions during application on the different plots are reasonably similar. Ideally, there should not be any rainfall before the treatments have dried e.g. for about 2 hours after application.

Shortly before application the number of bees per m^2, and how the assessments are carried out, should be recorded. Where a toxic standard has not been used, a foraging density of ideally at least 5 bees/m^2 on *Phacelia* or 2-3 bees/m^2 on rape and mustard (for the crop areas given in section 1.5) should be recorded shortly before application in order to verify exposure. These figures should not be used as validity criteria on their own. Lower figures should be explained and considered with other evidence of exposure. When assessing exposure, it should be remembered that foraging density may be affected by the total area available but at the colony level it will be determined by the total number of bees foraging on the test plots. However, in other cases foraging levels need to be related to the specific conditions of the trial e.g. for less attractive crops and pre-flowering application of systemic compounds (where exposure is related to a more sustained period that takes into account the duration of flowering).

Application rates

The product should normally be applied at the highest rate recommended for the relevant field use. Lower application rates may be applied e.g. if the off-crop risk needs to be assessed (using drift rates of application) or when exposure on weeds in orchards are tested (ground deposition rates). Volume of application and nozzle type should be as recommended and should be reported. Normally a single application will be sufficient when using a standard attractive crop. Multiple applications (according to the GAP) may be appropriate in specific cases e.g. for sprayed compounds that have the potential to move to the flowers via foliar uptake.

Mode of assessment and recording

Meteorological data

Temperature and humidity should be recorded at appropriate intervals throughout the trial period either at the trial site or at the nearest official weather station. Rainfall and sunshine or cloud cover should also be reported.

Type, time and frequency of assessment

Type

The precise nature of the assessment regime used in a particular field trial will depend on its specific objectives. The following parameters should always be assessed: flight and/or foraging activity in the crop as given by the number of bees/m^2 (by monitoring a fixed area e.g. 1 m^2, or using transects in the crop, in both cases for a defined period); general behaviour of bees on the crop and around hives using a standardised

approach; mortality of bees (using dead bee traps and possibly also on water-permeable sheets placed in front of the hives and in the crop); colony status/development (including consideration of disease and *Varroa* levels) at test initiation and test termination. These should be regarded as the core endpoints, which are particularly relevant for the interpretation of all field trial results.

In some cases, according to the requirements of the study, it may be appropriate to also 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).

Time and frequency

Pre-application assessment: at least twice for mortality and flight activity (once for in-hive assessments); one should be carried out immediately before application in the case of spray applications during flowering.

Post-application assessment: field observations e.g. mortality and flight activity 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 (i.e. sufficient to cover one brood cycle). The precise assessment schedule will depend on the study objectives and will need to be sufficiently flexible to accommodate prevailing conditions (colony assessments in particular should not be carried out during unfavourable weather conditions). Additional assessments should be carried out if appropriate on treatment day. Assessments should in general be performed at approximately the same time of day (again, adjusted according to prevailing weather conditions if necessary), although in-hive assessments (e.g. brood and food storage) can be carried out at any time of day provided climatic conditions are suitable.

Assessments may be continued for longer intervals e.g. to assess colony development over additional brood cycles if initial effects are seen. They may also be extended to consider the possibility of delayed effects or delayed exposure from stored pollen/nectar but these are not standard requirements and should be considered in the context of the study objectives (residue analysis may indicate if residues are occurring in food stores). In such cases the hives used in a study may need to be removed from the test plots (i.e. after the end of flowering of the treated crop) in order to maintain them for further monitoring (e.g. condition of colonies including brood assessments). The 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 flowering crops present), in order that they are not exposed to different location-specific factors. The collection of untreated pollen and nectar from non-crop plants by the test colonies at this stage cannot be avoided and reflects normal field conditions.

Results

Repeat tests where control mortality is excessively high and also where effects in the toxic standard treatment (if included) are low*****. Control mortality needs to be considered in the context that natural (background) mortality in colonies can be highly variable. Also, if mortality in individual colonies is excessive e.g. due to diseases or other non-treatment related factors, these may be excluded from the analysis rather than compromising a particular test group, where this can be justified. Information on exposure can be obtained from the assessments of foraging activity. Other information may also be used to provide additional information about exposure e.g. palynological analysis of pollen from forager bees, pollen traps or combs and residue analysis of nectar and/or pollen.

Mortality, behavioural and colony assessment data must always be provided and any other data which is relevant to the properties of the product being tested. Adjustments may be needed for differences between colonies in pre-treatment levels of some parameters e.g. mortality and foraging levels.

***** The higher tier testing working group of the ICPBR Bee Protection Group will assess available data in order to provide more specific guidance on these points.

If appropriate, statistical analysis should be applied using relevant methods, which should be justified. However, 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 (this should be justified). It should also be remembered that individual hives are not replicates but that treatment effects should be considered on a plot by plot basis. Whether statistical analysis is available or not, expert judgement will be needed to assess the biological significance of any effects seen in the context of each colony and the test conditions. This will also be needed to consider the relative importance of the various parameters assessed, in the context of impact on overall colony health and the specific aims of each study.

Conclusions

While it is considered that the current assessment of pesticide risk to honeybees conducted for EU regulatory evaluations is robust and effective, it is also recognized that a continuous process of review and development is appropriate. This allows feedback from the increasing wealth of experience that has been gained over many years of implementation to be used to improve the testing and assessment. In particular, this experience has identified areas such as brood effects and systemic activity where increased emphasis may be needed, in part due to developments in the methods of plant protection. In this context, the ICPBR Bee Protection Group set up a working group to review the higher tier testing methodology provided in the EPPO standard PP1/170. It was considered that this should provide sufficient information to allow appropriate tests to be conducted and evaluated. However, it was also recognized that there are a number of different routes from the Tier 1 risk assessment level that can trigger higher tier testing and so it is also important that there should be sufficient flexibility to accommodate the specific needs of individual tests. The proposed revision of honeybee higher tier testing presented in this paper reflects the considered view of the 10th ICPBR Symposium and will be submitted to EPPO for consideration in their review process. It should however be recognised that refinement and improvement of the test guidelines is an ongoing process using feedback obtained and a consensus approach within the ICPBR Bee Protection Group. In this regards the higher tier testing working group will report back with any further recommendations considered appropriate e.g. in relation to acceptability thresholds for control and toxic standard mortality.

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Proposal of the ICPBR Bee Brood Group for testing and assessing potential side effects from the use of plant protection products on honey bee brood

Roland Becker (BASF), Christine Vergnet (AFSSA), Christian Maus (BCS), Jens Pistorius (JKI), Ingo Tornier (Eurofins GAB), Selwyn Wilkins (Fera)

Introduction

There have been recent developments in toxicity testing of plant protection products on honeybee brood. These need to be assessed in terms of methodology and for suitability for inclusion in a sequential risk assessment scheme. According to EU Council Directive 91/414 EEC, regulatory testing and risk assessment on bee brood is required only when bees are exposed to IGR (Insect Growth Regulators) like pesticides. However, bee larvae may also be at risk by exposure to other types of substances. Concerns have been raised that the current testing and risk assessment schemes for honey bee brood fully address issues. As a consequence, a working group (participants are the authors of this paper) was constituted at the ICPBR meeting in York in October 2005. The remit of this working group was to evaluate recent methodological developments on honey bee brood testing and risk assessment and to integrate these into the current risk assessment where appropriate.

Background

Larval development or brood success is a vital part of the survival and/or productivity of honeybee colonies. As an overall protection goal it has to be assured that there are no unacceptable effects on bee brood impacting colony vitality. However, no specific trigger values for certain endpoints and for effects on bee brood have been established or are commonly recognized. Expert judgement is still an essential tool and basis of risk assessment.

Analysis

The spectrum of plant protection products that are relevant for bee brood testing are IGRs and other substances showing a higher intrinsic toxicity to larvae than to adults. Compounds with a high intrinsic toxicity to larvae as well as to adults are already covered by the current risk assessment and testing scheme. In order not to overlook unintentional side effects on larvae, PPPs showing pronounced larvicidal activity/effects on juvenile stages of insects (based on available screening and efficacy data and the results of non-target arthropod testing) should also be considered.

Recommendations

Two recent methodological developments - the *in vitro* laboratory bee larval test (Aupinel et al., 2005) and the bee brood semi-field test (OECD Guidance Document 75) - have been additionally considered by this ICPBR working group for integration into the risk assessment.

In terms of methodology, the ring testing of the *in vitro* laboratory larval test is still ongoing. However, once ring testing of this method is completed it may be considered as a tier I method in order to test pesticides for intrinsic larvicidal effects. In order to implement this test in the risk assessment scheme under consideration of the relevant endpoint, and for determination of relevant exposure figures and definition of appropriate TER values (Toxicity/ Exposure Ratio), a validation versus higher tier testing will have to be conducted.

The existing brood testing method (Oomen et al., 1992) method was designed to test for intrinsic larvicidal effects and is based on an unrealistically severe exposure of honeybee colonies to the tested compound. Therefore, it is considered as a type of intermediate tier brood test employing more realistic conditions than a laboratory test, but not as a semi-field or field test. For the time being the Oomen et al. method should remain as one option for testing of bee brood. Although this method was never formally validated, it has in almost 15 years of use proven to be a reliable tool to detect intrinsic larvicidal properties of compounds.

The semi-field brood tunnel test (OECD Guidance Document 75, based on Schur et al., 2003) provides a more realistic worst-case by exposure of the bees to a treated crop. This method was validated for spray products by ring-testing and had been accepted as an OECD Guidance Document in 2007. It can be used in the tiered testing scheme as higher tier test and should be integrated into the revised version of EU Directive 91/414 EEC as a test for bee brood evaluation.

In any case, field trials should remain the highest tier within the sequential honeybee risk assessment scheme for testing of brood effects. In order to address specific brood effects, available evaluation methods on brood development should be integrated into the field trial design. As established in the tiered honeybee risk assessment scheme, results from lower-tier studies are superseded by higher tier results, and lower-tier studies can be omitted, if higher tier testing is carried out initially.

Conclusion

The established sequential tiered risk assessment scheme for honeybees has proven to be successful concerning the protection of honeybees. The aforementioned proposals concerning evaluation of potential effects to bee brood should be integrated as a refinement of the current EPPO risk assessment scheme. The aforementioned recommendations of the ICPBR Bee Brood Group were established in consistency with the recommendations of other ICPBR working groups (Risk Assessment for Systemic Compounds, and Higher-Tier Testing) and give some guidance for a harmonized risk assessment scheme at the European level.

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France's proposal for Guidelines about setting Maximum Residue Limits in honey

Patrice Carpentier*, Anne Marie Chapel*, MariePierre Chauzat*, Bernard Declercq, Robert Delorme, Axel Decourtye, Alexia Faure*, Hervé Giffard, Fanny Heraud*, Jerôme Laville*, Anne Claire Martel*, Guy Milhaud, Eric Truchot*, Annick Venant,* Claude Vergnet*

* Afssa, 10 rue Pierre Curie, 94704 Maisons-Alfort Cedex, France

Abstract

Background: Honey is produced in an environment potentially polluted by different sources of contamination, so it is necessary to set Maximum Residue Limits (MRLs). These MRLs should be fixed as low as possible in relation to Good Agricultural Practices (GAPs).

The guidance provided in this Draft Working Document gives advice on:

- when and for what kind of active substance a MRL has to be set in honey
- how to propose a temporary MRL for a given active substance
- how to design, prepare and realise supervised residue trials when necessary

Results: The proposed approach is based on using the available data before an active substance or product is registered, and is divided into several successive steps, represented in a global decision-making scheme. The MRL will be set depending on the results obtained at each different step.

Besides, the applicants will have the choice between different methods to set a provisional MRL in pre-registration.

Conclusion: The initial proposal was a protocol on field residue trials proposed by Germany. The approach used in this guidance document proposes also other possibilities for fixing MRL without conducting systematically field trials. This proposition will be discussed at European level.

Keywords: Regulation 396/2005, MRL, honey, plant protection product

Introduction

Within the framework of Regulation 396/2005, guidelines relating to setting Maximum Residue Limits in honey have to be written. France was designated by the European Commission and other Member State to take in charge the writing of this guidance document. The French Ministry of Agriculture (the risk manager) asked Afssa (French agency in charge of the risk assessment) to make a proposal. In October 2007, the 'Working Group Afssa-MRL in Honey Working Group' was created, with the aim to propose a document for the end of 2008 that will then be submitted to the European Commission and other Member States.

Bees mainly produce honey, but also wax, pollen loads, propolis and royal jelly. Although these latter three are products for human consumption, their consumption is of low importance and honey remains the main beehive product used as food. The average consumption of honey per capita and per day in Europe is quite low (less than 5 g/capita/day) and represents a very small part of the total diet (between 0.04% and 0.17%). This would consequently not imply a significant contribution to the Total Maximum Daily Intake (TMDI), usually calculated in order to assess the chronic risk of dietary exposure.

Considering the acute risk, according to the EFSA Model for risk assessment of pesticides MRLs (PRIMo, Pesticide Residue Intake Model) and the lowest ARfD established today, it appears that, when there is no use in Europe, a default MRL set at 0.01 mg/kg is sufficient to guarantee the consumer safety. Otherwise, consumer risk assessment related to the consumption of honey will have to be evaluated.

To propose a residue definition

Honey is made mainly from nectar that is partially modified by bees and so by enzymes of animal origin. As a consequence, it appears that a specific residue definition should be established for this commodity, but, as

honey consumption should have little impact on TMDIs, if no specific metabolism study has been undertaken, the following definition of residue in honey is suggested as a default approach for monitoring and risk assessment:

Residue is the sum of parent and/or of all metabolites included in the residue definition for monitoring in plants and foods of animal origin.

To focus on potential exposure

Veterinary medicinal use

When an active substance is already used for beehive treatment (mainly to control bee diseases or parasites), this use is considered as a worst case, as the product is generally applied close to bees and honey. In that case the MRL defined under Council Regulation (EEC) No 2377/90 applies.

Intended Use

Crop attractivity, melliferous capacity: A given crop is more or less attractive to bees according to availability, quantity, quality of pollen and/or nectar (as well as that of honeydew). Moreover, the melliferous aspect of the crop has also to be considered. Indeed, even if a crop is attractive to bees, no residue will occur in honey if it is not melliferous.

Application before or during attractive periods (flowering, honeydew): the application period has to be considered to assess the exposure of bees to residues and then the risk of honey contamination.

'Residue in plant' properties

Systemic activity: systemic activity of the compounds included in the plant residue definition (active substance and/or its relevant metabolites(s)) has to be considered.

Residue level in aerial part of the crop: Depending on the residue level in aerial parts of the crop (if possible in flowers) or in honeydew, and on the physico-chemical properties of the compounds included in the plant residue definition, no further data may be necessary. It is considered that if the residue level measured in aerial parts of the crop is below 0.05 mg/kg, then the residue level expected in honey is assumed to be below 0.05 mg/kg. Therefore a default MRL of 0.05 mg/kg is fixed, based on a transfer factor of 1, that could be considered as conservative compared to data available in the literature (values from 0.0065 to 0.25 [1]).

To propose a choice of methods to fix a provisional mrl in pre-inscription

If the residue level is above the trigger value of 0.05 mg/kg it is necessary to propose a MRL, so that honey likely to contain residues may be marketed. Different options are proposed:

- considering data on residue in aerial parts of the crop,
- considering data from studies on transfer from syrup,
- considering data on residue stability in honey,
- considering data from field residue trials,
- considering monitoring data.

Use of data on residue level in aerial parts of the crop

Only data from aerial parts sampled during the attractive period of the crop or its weeds can be used (two to four trials could be considered sufficient). Aerial parts of the crop include leaves, flowers or nectar (grains are not considered). Based on a transfer factor of 1, an MRL proposal could be made with a suitable rationale. However, in order to set the MRL at a level as low as possible, analysis in flowers or nectar could be required if the residue level in leaves or whole plants is higher than 0.5 mg/kg.

Studies on transfer from syrup to honey

Spiking syrup used to feed bees has to be performed with compounds included in the plant residue definition at a level close to the one measured in aerial parts of the treated plants. If the residue amount in honey produced is lower than 0.05 mg/kg, then this value could be considered to propose a MRL.

If residue level is higher than 0.05 mg/kg, a MRL will be defined by extrapolation from data on transfer from syrup to honey (if these data are considered relevant).

Then a provisional MRL could be set from the Highest Residue (HR) [in plants] x average transfer factor (from syrup to honey).

Trials on the residue stability in honey

In order to check the stability of the residue in honey, the following method is proposed:

- Honey is spiked in triplicate with compounds included in the plant residue definition at a level corresponding to the highest residue measured in the aerial parts of the crop.
- Residues (compounds included in the honey residue definition) are quantified using a validated method on honey (according to document SANCO 3029 [2]) after a storage period of one month at room temperature (around 20°C).

If the residue level measured after one month under these conditions is below 0.05 mg/kg, a default MRL is fixed at 0.05 mg/kg.

If the residue level is higher than 0.05 mg/kg, a MRL could be proposed by extrapolation from stability data at the level measured in honey.

Use of monitoring data

Data from monitoring studies from non EU countries (if available) can be considered when the residue level is supposed to be higher than 0.05 mg/kg in plants, and to propose an MRL. These monitoring data may be obtained from different study plans, but a certain number of points must be addressed:

- The data concerning residue levels in honey (according to residue definition in honey) should reflect exposure of honeybees to residues,
- The data should be representative of critical exposure situations,
- The data have to be representative of different geographic areas and/or foraging activity of bees during foraging of nectar from treated plants,
- Statistical analysis of the results has to be performed and a statement on the reliability of the proposed MRL should be established.

Field or tunnel residue trials (allowing production of capped honey, and determination of the residue level in honey)

Tunnel or field trials are considered as the best way for studies to define MRL in honey. These trials can be performed using open field design or using tunnels (in the latter case the main condition is to obtain capped honey).

Results

See the decision making scheme for MRL setting in honey (see diagram).

Conclusions

From the entry into force of the EU so called *MRL regulation* (regulation CE 396/2005 [3]), MRLs of pesticides have to be set on new commodities, and honey is one of them. As a consequence, new guidelines have to be proposed to define MRLs on these commodities. So, the EU commission asked France to propose an approach to set MRL in honey. Considering that human exposure to plant protection products residues via honey is of little incidence, the approach proposed by Afssa[4], aimed to be as pragmatic as possible, is based on all available data and knowledge already acquired on a given active substance and its degradation products. This approach resulted in a draft document that describes a stepwise approach in order to propose a provisional or final MRL in honey. This document remains today a proposal and has no legal value, neither in EU, nor in France. France sent this proposal to the EU commission and then it will be discussed and amended in a near future, and may result in a guidance document in the coming years.

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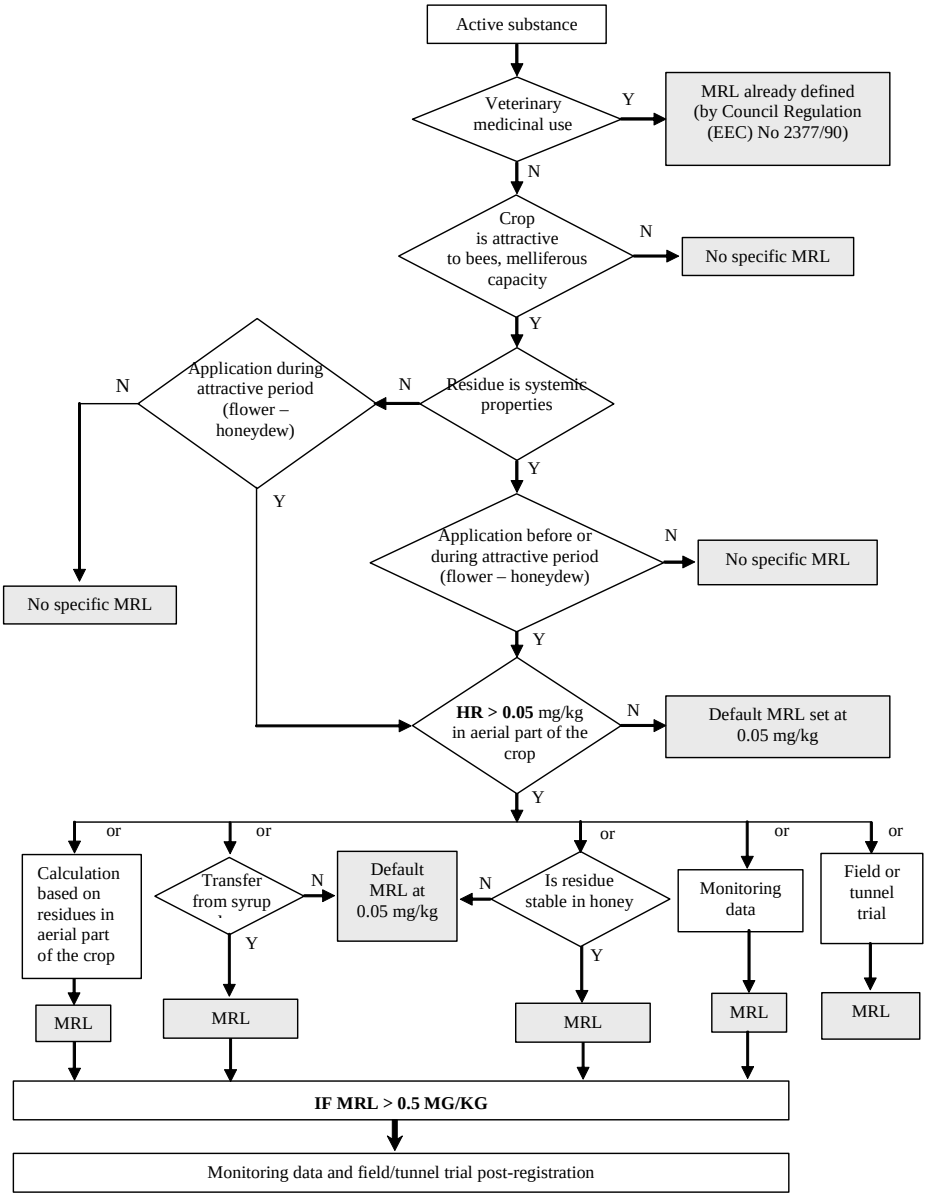

MRL : Maximal Residue Level – HR : Highest Residue

Diagram Decision making scheme for MRL setting in honey, as proposed by Afssa[4].

Plenary discussion on revision of the EPPO guidelines/risk assessment scheme

H.M.Thompson

Dusts from seed treatments

Oomen, chairman: The dust issue is likely to be a realistic risk also in countries other than Germany, France, Slovenia and Italy. Therefore should the meeting recommend to regulatory authorities in other member states that they should consider this risk?

Forster: In Germany the seed quality approach is in principle the same as in France. Dusts will be limited to 4g/100kg seed or lower, the sowing technique is amended to prevent the spread of dust and to reduce dusts as much as possible. The amount of dust should be reduced by 99% by these two methods; a risk assessment then can be conducted based on 1% dust to identify whether there is still a potential risk.

Oomen: Then the statement should be phrased that it is a general recommendation on behalf of the ICPBR symposium to the authorities in the EU to be aware of the potential risk and to take measures to reduce the risk from dusts from seed treatments liaising with BVL, JKI and AFSSA on appropriate approaches to the issue.

Forster: Seed trade is very flexible in the EU and so imported seed can be used if the exporting country has authorisation. There is therefore a need to ensure that all EU member states have a similar level of quality for seed treatments.

Alix: The risk manager needs to be included in discussions as well as the risk assessor to ensure that appropriate batch analysis rules are in place and to encourage the adaptation of machinery.

Lortsch: Will these minutes be in draft and circulated?

Oomen: Only the final minutes will be published.

Thompson: The organising committee will review the draft minutes.

Lewis: France and Germany have significant expertise in this area of seed treatments; we need to ensure that the information and guidance is passed to authorities.

Oomen: Is it a good idea for the ICPBR meeting to act as intermediaries?

Nienstedt: The Commission and member states have been informed of the issue.

Forster: They are aware of the incident but not aware how the problem is being addressed. The example of dust in seed bags can be used to highlight quality criteria with respect to dust in bags and the redesign of sowing machines should also be considered.

Brasse: France and Germany have addressed the issue but Italy reported a similar issue in 2002 and this has not been addressed there - the information needs to go to the governments.

Giffard: In France the issue has been addressed for dusts but not for the redesign of sowing machines.

Oomen: *The conclusion of the meeting is that the risk of dusts from seed treatments should be brought to the attention of authorities in all member states and the solutions being developed in France and Germany brought to their attention.*

Revision of the EPPO guidelines and risk assessment for honeybees

Oomen: The role of the ICPBR Bees and Pesticides Group is to address problems as we perceive them and to use working groups to develop proposals. There is a need to integrate the three working group proposals highlighted at this meeting into the existing EPPO risk assessment scheme and to develop these as a new scheme. We therefore need to discuss these three proposals.

The field and semi-field testing guideline

Kievits: On the tunnel effects there is a need for a toxic standard for compounds more toxic than dimethoate.

Lewis: It is important to be clear what the toxic standard is for – it is only to ensure the system is working and to show sensitivity and it is not intended to compare effects relative to the treatment (this view was endorsed by Coulson).

Kievits: What is the toxic standard for toxic contaminants in nectar – for a systemic compound?

Becker: You need to distinguish what the test is for, an IGR, a sprayed product or systemic effects and design the test appropriately.

Alix: For systemic compounds it is more difficult, due to the exposure issue - the residue is transferred to nectar and this varies by crop type and even by variety. This is why the toxic standard is used to show the study design has worked and the system is sensitive. A toxic standard for systemics is then very difficult to identify. Therefore we use residue analysis to check exposure to the treatment for systemic compounds.

Candolfi: When setting the validity criteria for the control and toxic standard you need to insert a concrete figure or omit the approach of validity criteria.

Coulson: This was discussed and the group didn't consider setting a fixed number for validity criteria for the toxic standard - addressing this may be an action of the meeting.

Lewis: Critical analysis of the acceptability of the study is required.

Candolfi: *There is a need to analyse data for toxic standards from existing studies.*

Coulson: *Agreed. The group should take action to look at developing a database, with a view of developing validity criteria of field and semi-field tests.*

Stevenson: The need for expert judgement in analysing data has been identified, e.g. an experienced beekeeper.

Brasse: Which country would allow the use of a toxic standard in a field study? This use should be avoided.

Coulson: Felt he couldn't make the statement but the meeting was able to raise this issue.

Kievits: Tunnel tests can't assess issues with contaminated pollen which may be stored, fermented and consumed weeks or even months later.

Coulson: The intention is that the study takes into account the chemistry of the molecule and the test is designed to address the issues raised.

Bruneau: The quantity of pollen from a tunnel test is low and additional pollen after the test is conducted is likely to dilute this further.

Coulson: How could this be addressed?

Bruneau: By using a PEC/PNEC approach.

Oomen: The use of laboratory or model derived data raises the issue of extrapolation to the field while a field test addresses this directly.

Bruneau: The field test should be repeated to ensure the results are statistically valid.

Oomen: The honeybee risk assessment scheme is the only EPPO scheme which has been validated and data over many years has shown it to be reliable.

Bruneau: This is true for sprays but the problem is with systemics.

Alix: *You may have delayed exposure with pollen – we are aware storage may occur - but this is reflected in the duration of the study. Studies cannot represent every condition; they aim to determine that effects do not occur under the conditions of a field study which cannot cover all situations. A wider range of conditions of use is only possible through monitoring and this is currently occurring in France to help to confirm the risk assessment is correct.*

Bakker: The strong technical guidance on cage size in the semi-field study may be too restrictive - smaller cages can be useful and the paragraph needs rewording.

Coulson: This guideline is for standard tests – we are supportive of smaller cages for special designed studies.

Lewis: *Smaller cages are recognised as useful for addressing specific questions.*

Giffard: Larger cages are needed, smaller cages are OK for acutely toxic insecticides but not for colony studies, sizes need to increase to 100-150 m^2.

Coulson: The minimum was set for the standard study but the guidance allows for smaller cages for specific studies. Is 40 m^2 sufficient?

Brasse: There is a need to relate the size of the colony used to the area of forage available, but you also need a minimum size of colony to see effects.

Lewis: There is a need to balance the level of detail in the guideline with being too prescriptive.

Pistorius: There is a need to identify the colony size by size of frames and a rough estimate of numbers of bees and brood cells.

Wallner: Some data suggest for fields that the minimum area is 3 ha and, rather than monoculture of *Phacelia*, that clover is sown to flower at the same time to ensure mixed forage and thus ensure attractiveness to bees.

Tornier: For winter oilseed rape the minimum is 1 ha with 4 colonies, this is the same stocking rate as a commercial beekeeper uses. *Phacelia* is much later flowering, in central Europe mid June-July, and there are very few other flowering crops which reduces alternative forage.

Bruneau: There has been a problem with *Phacelia* in Germany in that the bees don't only forage on the *Phacelia*.

Tornier: The *Phacelia* needs to be irrigated to ensure it is attractive as forage for bees.

Maus: For systemic compounds the effect on honeybees is addressed in the real crop, not *Phacelia*.

Karise: *The guideline should state that it is ensured there are no large apiaries nearby rather than recording their presence.*

Coulson: Agreed.

Lortsch: What is the definition of behaviour?

Coulson: Behaviour within the tent or field.

Laves: You are not assessing the foraging behaviour of the bees in the guideline but the number of bees per m^2; they aren't necessarily foraging.

Coulson: The word foraging should be removed and replaced with number of bees/m^2.

Lewis: Appropriate behavioural observations need to be made for the bees on the crop.

Forster: You still need to know the number of foraging bees.

Lewis: You need to count the numbers of bees on and above crop but if the behaviour is to be recorded you need to know what the bees are actually doing.

Lortsch: We do not agree to the field and semi-field schemes as it is not a final text for systemics.

Alix: *It is a wide framework which highlights which observations should be made, based on observations at earlier stages of testing so that the study design is tailor made to the issues to be addressed.*

Lortsch: This answers my concern.

Brasse: The guideline is not a word-by-word guide, it needs to leave areas open to allow the design of the study to address a specific question.

Nienstedt: *We need to ensure that the terms methods, guidance documents and guidelines are not mixed.*

Zlof: It may help if I provide an outline of the EPPO procedure for guidelines. EPPO is an international organisation founded in 1951, there are 50 member countries, the whole of the EU, Russia and the ex-Soviet countries and North Africa. The remit relates to phytosanitary regulations and plant protection products with the aim of facilitating regulators in authorisation and industry in registration by harmonising procedures. The Environmental Risk Assessment Working Panel published a scheme which covers all aspects of the environment, soil/water and organisms in 2002-2003. Member countries have recently asked EPPO to revise the standard and risk assessment scheme for honeybees. Schemes are adopted into national legislation and EPPO is meant as the minimum requirements. All documents are developed by specialist groups of experts, not national representatives. They are then sent to the member countries for comments which are reviewed by the experts and then the documents are sent to the Working Party on Plant Protection Products. The aim is for the honeybee guidelines and scheme under discussion here to be approved in May 2009. I would like to thank the organising committee and working groups for their activities.

Systemic risk assessment

Candolfi: Can the data from which the TER values were calculated be seen?

Alix: We started from acute toxicity data in the Agritox database and a high default residue level of 1 mg/kg for pollen and nectar. We then made the TER calculations for all substances whatever the mode of action. We compared the 48hr LD50 with the 10 day LC50 (UK PSD funded study) and a factor of 10 covers the range of values. We tested the relevance of the trigger values in the TER calculations. The proportion of substances going to tier 2 for all compounds based on 1 mg/kg and a trigger value of 10 was 16% and included almost all insecticides, so this was a check for false positives. We also checked that the only insecticides that passed, were the IGRs and non-bee-toxic insecticides. We will provide the background in the proceedings.

Kievits: The TER of 10 is too low, we propose better screening at tier 1 including persistence – we have to find a better method of screening.

Alix: Persistency is included in the scheme, even if it is not directly related to the treated crop, e.g. effects in a following crop. The safety factor is based on an acute effect in the right species (we are not trying to extrapolate to another species) and the concentration in nectar and pollen we are using (1mg/kg) has never been observed at this level and is therefore extreme. The concern is actually only for a few substances, many of the insecticides are toxic but not systemic. We therefore need to ensure we don't over-cover the systemic issue so as to be consistent with the rest of the Ecotox risk assessment. For the substances of concern these are all triggered as values are well below 10.

Oomen: Is there agreement with the proposal of the working group on systemic risk assessment?

Kievits: We have a lot of points of concern but agree with the global structure of the risk assessment; our concerns will be sent direct to the working group.

Alix: *Please send each concern, illustrated with data, in a table format to allow a response on each issue.*

Brood effects

Oomen, chairman: Are there questions for the brood group?

Barrett: There is stated lack of validation data for the Oomen method, the bee brood feeding study has been used for many years by many labs and we need to pull together data into a single place to show the level of use and data available.

Becker: I agree this is a good way forward.

Barrett: It would be a powerful database for a range of compounds and doses.

Becker: *The working group wants to reconsider the Oomen method (bee brood feeding studies) including reviewing such data.*

Bruneau: The trigger of 30% decrease in brood levels in a tunnel test - where does the data come from?

Becker: There is a need for expert judgement on this as lower levels of decline may be an issue and may trigger a need for a field test.

Barrett: Under what conditions would the test be needed – for all products?

Becker: Not for all products but for IGRs and systemics, or if data from efficacy or ecotox studies show effects in juvenile or larval stages of arthropods.

Oomen: Do we agree to the proposal of the working group on brood effects?

Lortsch: Long term effects are not covered in the proposal.

Becker: The effects to 7 days are in the tunnel but evaluation is up to 28 days and can be extended through 2 brood cycles, this is the same as for field studies for long term effects.

Bruneau: There is still the problem of diluting effects of other pollen sources in the tunnel.

Tornier: I agree for the tunnel, this is why there are control and positive control treatments.

Bruneau: The variability is high, so results are not significant - so what does an effect mean?

Tornier: Do you have a proposal for an alternative?

Becker: Tunnel tests are not always perfect. There are likely to be a number of tests and together these are unlikely to completely mask effects. If there is still concern there is still the option to monitor colonies in real use.

Alix: The duration of the flowering is the exposure driver, but bees will also forage elsewhere. Therefore we maximise exposure by placing the bees within the test field for the complete duration of flowering.

Becker: If there are still concerns please send them to the working group.

Bruneau: Agree.

Oomen, chairman: *The working groups will consider the points raised and the final versions of the schemes will be circulated in mid January for final comment (2 week deadline) prior to submission to EPPO.*

He then thanked the organisers and participants and closed the meeting.

II. Test and risk assessment (incl. systemic effects, field testing, bee brood)

Risk Assessment of Pesticides and the role of EFSA

Karin M. Nienstedt

EFSA, Largo N. Palli 5a, Parma, Italy

Abstract

The European Food Safety Authority (EFSA) was created by the Regulation EC 178/2002 on 28 January 2002 with the mandate to provide scientific advice and support for the European Community policies in all fields with impact on food and feed safety. The PPR Unit (Plant Protection Products and their Residues Unit, Risk Assessment Directorate) as well as the Pesticides/PRAPeR Unit (Scientific Cooperation and Assistance Directorate) both works on Plant Protection Products in relation to Directive 91/414 EEC. PRAPeR coordinates the Pesticide Risk Assessment Peer Review for the approval of active substances by the European Commission and the Members States, whereas the PPR Panel provides independent scientific opinions and guidance for the Community's legislation in the field of plant protection products.

Actual examples have been presented regarding the role, working procedures and results of the PPR Panel and PRAPeR in relation to the risk assessment of plant protection products to bees (e.g. EFSA-Opinions, EFSA-Conclusions). Information on on-going and scheduled work of the PPR Panel in this area have also been mentioned. In line with EFSAs commitment for transparency, details of the ongoing work are published on www.efsa.europa.eu.

Systemic plant protection substances and products: how to assess the risk for bees? A beekeepers point of view

Etienne Bruneau[1], Janine Kievits[2], José-Anne Lortsch[3] , Szaniszlo Szöke[4]

[1]Centre apicole de recherche et d'information, 4 place Croix du Sud, 1348 Louvain-la-Neuve, Belgium
0032 (0)10 47 34 16 (bruneau@cari.be)
[2]Inter-Environnement Wallonie, 6 boulevard du Nord, B-5000 Namur, Belgium - 00 32 (0) 81 25 52 80
[3]Syndicat national d'apiculture, 5 rue de Copenhague, F-75008 Paris, France
[4]Agronomist and beekeeper, 16 rue de Brionsart, B – 5340 Gesves, Belgium

Abstract

Background: The current plant protection products (PPPs) assessment is no more suitable when applied to systemic substances since systemic chemicals can contaminate nectar and pollen during a long length of time. Largely focused on the acute toxicity, the current assessment scheme does not take into account several elements i.e. the chronic toxicity, the possible synergies between substances, and between pathogens and PPPs. Possible bee contamination through nectar and pollen leads to a specific exposure, mainly oral, concerning the hive bees, including larvae, drones and queens, as well as potentially delayed through the stored honey and pollen consumption. Moreover, regarding the long-term exposure, sublethal chronic effects should be taken into account.

Results: For such substances we would take both the chronic toxicity and the acute toxicity measurements into consideration. Therefore the TER should be calculated based on the lowest LD_{50} and in the case of risk, the PEC/PNEC ratio should be measured and calculated for various behaviours. A larvae test should also be performed. Tunnel tests may be helpful but the exposure to the PPP cannot be proven and the bee behaviour observation is currently inaccurate. Further research on the effect of small doses of PPP on the bee immune system seems more than necessary.

Conclusion: A new assessment scheme, which takes these parameters into account, is the core of our contribution.

Keywords: Assessment scheme, chronic toxicity, sublethal toxicity, synergies, larvae test, PEC, PNEC, TER.

Introduction

Like all other pollinators, honeybees are gathering everyday thousands of micro-samples from their environment. Because of their wide foraging area and their intense foraging activity, they are already used as bio-indicators (Porrini et al. 2003).[1]

Moreover, the honeybee has fewer genes encoding detoxifying enzymes than other arthropods. Therefore, bees seem to have less capability of detoxification than most of the other species of insects (Claudianos et al. 2006).[2]

The bee colony, as a super-organism, can survive only if key-pheromone relations and numerous complex behaviours are preserved.

Depending on the flowering, the bees perform sophisticated foraging strategies, which can vary from year to year, influenced by the temperature and the rainfall conditions. Food is stored for long periods and the consumption of harvested nectar and pollen can therefore be delayed of several months. Finally, the colony is composed of different classes and castes of bees and the toxicity of a single substance therefore varies between classes and casts.

These three facts demonstrate by themselves the importance and the difficulty to perform an accurate assessment of the PPPs.

The current plant protection products assessment does not satisfy us entirely, especially when applied to systemic substances. Systemic chemicals can contaminate nectar and pollen, during the entire blossom. Largely focused on the acute toxicity, the current assessment scheme does not take into account several elements such as the chronic toxicity, the toxicity variation between bee classes and bees castes, the possible synergies between substances and the possible synergies between pathogens and PPPs.

The objective of this document is to show alternatives to the definition of an assessment scheme, in order to consider the specificity of systemic substances and PPP particularly when they are suspected to contaminate pollen and nectar.

Specificity of the bees exposure to systemic PPPs

Opposite to sprayed non-systemic products, the systemic products, particularly when used as seed or soil treatment, lead to a different and specific bee exposure. This specific exposure is described in some scientific publications (e.g. Alix and Vergnet, 2007).[3]

To be very clear in this exposure we would remember the main conditions:

- Honeybee is exposed through their feed sources, nectar and pollen, and not through the drift when flying. It would appear to be mainly an oral exposure (opposed to sprayed PPPs leading mainly to a contact exposure).
- The contaminated food is brought back to the hive where it will be used by the whole colony. The food can contaminate all casts: workers, drones and queens and all the other classes: nurses, storekeepers, foraging and winter bees.
- The nectar and the pollen brought to the beehive will be stored. The nectar can be used immediately. On the contrary the pollen requires a one-week fermentation to be digestible by the bee. In addition, the stored food will be consumed during the periods of the year outside harvest time and particularly during wintertime. Thus a pollen collected in August may be consumed the following March or even early April; the consumption being delayed by up to 8 months.

- Considering the flowering timescale and the possible storage contamination, which will later be consumed, the actual contamination timeframe can be extended to long periods (opposite to sprayed products which are generally quickly downgraded by photolysis). Possible contaminations can thus have chronic lethal and sublethal effects. If the PPP is a neurotoxic, the sublethal effects may concern any behavioural pattern.

We add to the above that some of the concerned PPPs are already found in significant concentrations in the environment (Chauzat et al. 2006).[4]

A new assessment scheme

Given the specificity of the honeybee's exposure to systemic substances when present in the foraged matrices, it is necessary to develop a new assessment scheme. We argue it will not be relevant to adapt the current scheme for many reasons:

- The "trigger value" (Hazard Quotient =HQ > 50) is not entirely relevant.
- The current higher tier tests are not sufficient to assess effects.

The current higher tier tests are not sufficiently reliable since the chronic effect assessment needs long-term effects assays.

The trigger value

Relevance of the HQ for systemic PPPs used in seed soil treatment

The HQ coefficient is only validated for products used in sprays (SanCo 10329, p 18).[5] The HQ takes into account the acute, oral and contact LD_{50} only, as well as the application rate. The persistence and the chronic toxicity, which are both essential parameters to appreciate the risk level of systemic PPPs are not taken into account.

Villa et al. (2000)[6] propose a method for assessing the risk of contaminated pollen via a TER (Toxicity Exposure Ratio) calculation based on physical and chemical properties (P_{oa}[1]), persistence, application rates and LD_{50}. This study concludes that the comparison between the two approaches (TER and HQ) shows a relatively good result but points out that chemicals with a high $logK_{oa}$ are classified as more dangerous by TERs in comparison to HQ.

Even for a sprayed product, the HQ reliability seems to be low when the substance is systemic.

We can make the conclusion that the systemicity must be estimated at the first tier assessment, both for sprayed products and for products used as seed or soil treatment.

Obviously this does not mean that a high HQ matches with a small risk for honeybees when the product is systemic. A high HQ should always be a warning about the PPP risk for bees because it indicates that the spread amount is high compared to the acute toxicity.

Elements to consider at the first tier step

Every evaluation of PPP risk for honeybees should start considering the following points:

- The acute LD_{50} and the HQ
- The octanol/water (octanol/air) partition coefficient as systemicity indicator
- The persistence
- The presence of the substances and their metabolites in the foraged matrix, pollen and nectar. This presence must be detected by using analytical methods, which have the lowest limits of detection and quantification. Especially, these limits must be of the same order of magnitude as the toxicity of the substances on bees.

- The application type for the active substance: some substances used in soil or seed treatment are designed to protect the whole plant during its growth (e.g. insecticides in seed treatment). This kind of PPPs should always be considered as systemic substance

When the substance is considered as a systemic and persistent[2] product and /or when the PPP is used as seed treatment (to protect the whole plant), the trigger value should be a TER.

When the products and substances are present in the foraged matrix, the chronic toxicity (both lethal and sublethal) should be assessed; the TER should take into account the lethal chronic toxicity and, except when the TER shows a low risk, the sublethal effects are to be assessed.

For the sublethal effects assessment, the PEC/PNEC approach (predicted environmental concentration / predicted no effect concentration ratio) seems to be the only appropriated way today (Halm et al. 2006).[7]

Then the products and substances will be directed to a particular scheme as soon as the systemicity is attested.

Proposal for the first tier assessment

We would propose the following scheme:

First tier assessment: acute LD_{50} + systemicity

A substance is systemic:

- if (log P_{ow}, log P_{oa},) + persistence leads to a risk index > X
- if detected in the foraged matrices
- if applied as seed or soil treatment and aimed to protect the whole plant

Higher tier assessment

When low systemicity:

- and HQ < 50: no higher tier test

and HQ > 50: higher tier tests of current assessment scheme (cfr EPPO 170)[8] (bee brood feeding test, cage tests, tunnel tests, field tests).

Higher tier assessment

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- and HQ > 50: higher tier tests of current assessment scheme (cfr EPPO 170)[8] (bee brood feeding test, cage tests, tunnel tests, field tests).

Higher tier assessment

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- and HQ < 50: no higher tier test
- and HQ > 50: higher tier tests of current assessment scheme (cfr EPPO 170)[8] (bee brood feeding test, cage tests, tunnel tests, field tests).

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[1] P_{oa}: partition coefficient octanol/air. The log P_{oa} is directly proportional to the uptake by leaves of hydrophobic organic chemicals from the air. This study is concerned with sprayed PPPs absorbed by the plants leaves as vapour.

[2] for instance: log Pow < 5 et DT50 > 7days; log Pow is given to PH = 4, PH=10 and the smallest value is considered.

When the substance is considered systemic: chronic LD50 + bee exposure => TER calculation when the TER shows a low risk:

- and HQ < 50: no higher tier tests
- and HQ > 50: higher tier test of the current assessment scheme

When the TER shows a risk: bee brood feeding test and PEC/PNEC assessment.

TER calculation

TER = ratio between the LD_{50} and bee exposure (consumed substance quantities). The LD_{50}s considered are oral and contact, acute and chronic. The quantity of the consumed substance by the bee depends on:

- the substance concentration in the pollen and the nectar (measurements)
- the amount of pollen or nectar actually consumed by the bee (estimation)

For each TER, the quantity of the substance considered is:

- the amount consumed by the bee in real conditions, as it can be evaluated (CST report)[9] (Rortais et al. 2005)[10]
- the amount consumed within the considered LD_{50} timeframe (48H or 10 days)

When the bees have consumed both nectar and pollen of the considered plant, the amount to be taken into consideration should be the sum (amount consumed through the pollen + amount consumed through the nectar).

Acute toxicity

The acute toxicity is assessed following the current rules.

If the relation dose/mortality curve shows irregularities or variations at the trial replications, the safety factor should be greater than usual (TER_2>TER_1, cfr scheme below).

Chronic toxicity

The trial design is similar to the acute toxicity determination, except that the total dose of substance is divided into ten daily doses (from day 1 until day 10) given in the morning. If the relation dose/mortality curve shows irregularities or variations at the trial replications, the safety factor should be greater than the usually used one (TER_2>TER_1, cfr scheme below).

Chronic toxicity / acute toxicity ratio

Calculating this ratio is a way for assessing the potential accumulation of a substance. The chronic toxicity test cannot last more than 11 days in laboratory since the bees do not bear confinement. When the ratio between acute and chronic LD_{50} is greater than 2, the sensitivity to repetitive doses is more than twice the sensitivity to a single dose, meaning that a clear cumulative effect is observed. In such case, a greater safety factor should be used for the TER calculation.

Concentration measures in the foraged matrix

The analytic methods used should permit the concentration detection at a comparable level to the NOEC or LOEC considered during a long period of time; however the NOEC and the LOEC are not yet determined at this step of the assessment.

We would propose a limit of quantification LoQ ≤ LD_{50}/200.

For instance, a product for which the LD_{50} = 5ng/bee - that is to say 50ng/g of bee -, the limit of quantification must be at most 0,25ng/g (0,25 ppb). Such quantification limits are nowadays possible; methods are described, for instance by Bonmatin et al. (2006)[11] (LoQ : 1 ppb ; LoD : 0,1 ppb).

For the pollen, the current analytic method must include the dissolution or the grinding of the pollen envelopes because the toxic substances are located inside the grain and not on its surface.

We would emphasize too that it is not relevant to validate an analytic method by testing its ability to detect the substance when spread onto the pollen.

The pollen should come from pollen traps or better, from the flowers because this is the one consumed by the bee. The trap pollen needs to be sorted out when the studied contamination is linked to a specific crop. The comb pollen is usually a mix of different pollen sources, which conduct to contamination dilution. This fact should be taken into account when sampling and for the forthcoming test conclusions.

Quantity determination of pollen/nectar consumed by the honeybee

The quantities of pollen and nectar considered for the exposure estimate are of course the amounts consumed by the bee in real conditions. Many publications and reports estimate food quantities consumed by a bee or a colony. Concerning the pollen, 65mg is given for a nurse upon 10 days (Rortais et al. 2005)[10] or also 160 to 180mg for a worker during its whole life (Keller et al. undated).[12] Pollen amounts consumed by winter bees are unknown at this time. Every beekeeper knows that wintering may only succeed if the bee colony has collected important quantities of pollen during summer. Most of this pollen will disappear during winter and early spring: it has been consumed by the bees, and particularly by nurses for feeding the early brood. The winter bees are not numerous (10 000 – 15 000) and they will feed brood for a long period. This means that pollen consumption per winter bee is potentially more important compared to summer bees. Thus the pollen toxicity for winter bees should then be tested specifically. Before carrying out this test, it is necessary to quantify the pollen amounts consumed by winter bees with great care in order to define their exposure.

Concerning the nectar, Rortais et al.(2006)[10] quotes a range of 72,8 (wax foragers) to 898,8mg/bee (nectar foragers). Beekeepers can make a quick estimation: a colony harvests 60 kg of honey, that is to say 150 kg nectar during one month time (a usual average amount during the sunflower blossom). Then, two forager generations must be considered, or about 20 000 foragers (it is commonly considered that a hive contains about 10 000 foragers simultaneously). During its lifetime, each honeybee will harvest 7.5 g of nectar from which about 10 % is used for the forager itself. So each bee will consume about 750 mg in 2 weeks, or 107 mg in 48 hours. The main part of the nectar is not immediately consumed; it is brought back to the hive, and regurgitated to be stored into the combs, or shared with other bees of the colony. This part can generate a contact toxicity: it is not digested, however it is in contact with the oesophagus and the stomach of the honeybees. This contact toxicity is never taken into account in the current TER assessment. The consumed quantities taken into account should be pursuant with the principle of the *worse case* (harvested amounts in important honey-supplier crops as sunflowers e.g.).

Proposal for the trigger value

The trigger value takes into account a safety coefficient, which should cover the sublethal effects. This coefficient should vary according to 3 parameters:

- the steadiness of the results from replications
- the regularity of the mortalities curves
- the cumulative effect from doses (ratio between the acute and chronic LD_{50}).

A bad reproducibility of the results, or irregular mortality curves show uncertainties that should be covered by a greater safety coefficient; and sublethal effects appearance is more likely when this substance gets accumulated into the bee body.

The PEC/PNEC approach

The sublethal effects from the chemical ingredients on the bee behaviour or other useful insects are reported by a great number of publications (Desneux et al. 2007)[13]. For instance, sublethal doses of insecticides impair the waggling dance (parathion), the harvest and the transport of the nectar (diazon), the homing flight (deltamethrin) (Vandame et al. 1995)[14].

Regarding the honeybee, we find in the scientific literature remarks about:

- development
- survival, fertility and egg-laying capacity of the queen
- the mobility of the bee
- the bee capability to find its way on short distances (using the visual or olfactory memory)
- the bee capability to find its way on long distances (using the aptitude to find its way according to the position of the sun and the memory associated to that capacity)
- the behaviour when feeding and the training capacity
- foraging intensity
- thermoregulation

Moreover PPPs are likely to reduce the honeybee length of life (essential parameter for the harvest), its immune capacity, and other behaviours that are necessary to the integrity of the colony and its natural development, such as:

- the bee brood feeding
- the whole behaviour leading to swarming
- the combs construction and the balance between drone cells and worker cells
- the search for a new nest in the swarming period and the transmission of information to the other pioneer bees ….

A complete bee behaviour model does not exist today; we do not believe that such a model could be possible considering the behavioural complexity of this super-organism.

The question has sometimes been asked about the ecological relevance/reliability of the sub-lethal tests for the concerned effects on behaviour. Despite the fact that man has grown bees for a long time, their physical and behavioural characteristics have not been altered. All aspects of the behaviour likely to be affected have a utility in the survival and good development of the colony. We have no knowledge of any scientific element that would blame or deny this postulate.

The sub-lethal effects can vary according to several parameters

- The sub-lethal effect of a substance is not necessarily related to the dose. For instance Kacimi El Hassani et al. (2007)[15] notice that acetamipride, used as a topical application affects the bee locomotion activity at 0,1 and 0,5µg/bee but not at 1 µg/bee.
- The dose having sub-lethal effects for similar substance varies with the considered compartments: Kacimi El Hassani et al. (2007)[15] do not notice any effect from the thiamethoxam in doses < 1ng/bee on the mobility of the bee while a team of the INRA notices some effects of the same substance at 0,5ng/bee on the orientation (Belzunces L., 2008, personal communication)
- The effect can vary according to the age of the bees: Guez et al. (2001)[16] notice that sub-lethal doses of imidaclopride do increase the number of tests needed to remove the extension reflex of the proboscis by presentation of a sucrose solution for young bees (< 7 days), while for elderly bees (> 8 days), the number of essays necessary to create the reflex goes down during the first hour after treatment and goes up four hours later after the treatment.
- The effect can vary finally according to the season: Decourtye et al. (2003)[17] notice less LOEC of imidacloprid (conditioning of the extension reflex of proboscis) to the summer bees than to the winter bees, which seem therefore more resistant to the substance, according to this behaviour at least.

- We also notice that the effect whether contamination affects nectar or pollen can vary because the classes of bees are different. Contamination of the nectar will also quantitatively affect the foraging bees more than the nursing bees. The last ones are more affected by pollen contamination.

The predicted environment concentration PEC

The PEC is based on measurements of the substance and its relevant metabolites in the foraged matrices. This parameter has been established for different types of bees (males, workers, queens and among the workers: nurse bees, foraging bees), which allows taking into account the potential difference of exposure between the different categories of bees.

The predicted non observable effect concentration

The determination of the PNEC brings the necessity of making a series of tests to measure the lowest concentrations that does appear any effect (LOEC). The PNEC are the LOECs assorted by a coefficient of security depending on the accuracy and reliability of the tests. These tests should be made on the concerned categories of bees and on the concerned behaviour (for instance the foraging for the orientation).

Various methods appear in literature, which allow to determine the PNECs. Most of them are methods used in labs. They are concerned with bee brood (bee brood feeding test: Aupinel et al. 2007[18]), bee locomotion, homing flight (see for instance Vandame et al. 1995[19]), the learning abilities assessed through the proboscis extension reflex (Decourtye et al. 2004[20]), the bee thermoregulation, the foraging intensity. The bees lifespan and the queen egg-laying should be assessed too because these capacities are of high biological significance for the hive. The methods' strength is not always attested and some of these should be performed again in different laboratories in order to make or to elaborate ring tests that could be brought into the assessment scheme.

A particular attention should be paid to the effects on the immune capacities since such effects are documented for various substances and microorganisms, some non-pathogenic organisms becoming pathogenic when associated with defined substances.

Immune capacity

With some species of arthropods, the chemical PPPs at very small doses can make the individual sensitive to some pathogens, so that the economic use of such associations is taken into consideration (for instance for termites control). As far as we know, no study has been performed on this subject. However some pathogens (*Beauveria*, *Nosema*) and some substances (imidacloprid) are the same as substances and pathogens possibly present in the hives (Cuthbertson 2005 et al.[21], Feng and Pu 2005[22]). Moreover, some target pests are *Hymenoptera* (termites, leaf-cutting ants - Santos et al. 2007[23]). Pesticides with long-term effects are thus likely to depress the bee's immune capacity.

From another point of view, CCD mortalities seem to go with or to be due to various pathologies, appearing as abnormal in number and in intensity (*Nosema*, virosis….). During eco-toxicology tests performed by PPPs manufacturers, it is observed that treated hives suffer from *Nosema* development or from loss of queens at the end of the test. Researches should be undertaken on bees in this matter (foraging bees and home bees).

Relevance of the field and tunnel tests for systemic PPPs in soil and seed treatment

The assessment of systemic PPPs in tunnels or in the fields have to be made over a long period because these PPPs are likely to contaminate the nectar and/or the pollen. The observations may have to vary from several weeks (observation of the effects on the summer bees) to several years (Cruiser field tests in France). Particularly the evolution of colonies on contaminated stocks during wintertime is a multi-annual fact.

Tunnel tests

Assessing chronic effects requires bee colonies to be confined for a long period of time. Bees cannot bear being prevented from flying, what they usually do over long distances; the colonies development can be affected, such as reported in studies submitted in the authorization reports. For instance, the colonies are completely deprived of brood at the end of the test. This observation does not permit to conclude that the product is harmless since the effect can be masked by the confinement.

The tests in tunnels cannot control the exposure due to the differed consumption. When colonies are put in the tunnel with food frames, it is not assured that the pollen consumption during the test is provided by the contaminated source instead of the combs stocks. Only foraging bees are in contact with the treated pollen that they bring back to the hive, without any effect when the contaminated substances are inside the grains of pollen and not on the surface. Due to these reasons, tunnel tests cannot be considered as a higher level tests in comparison to PEC/PNEC tests when the substance is detected in the foraged matrices, particularly when no toxic standard is available.

Field tests

The tests on the field will raise a double problem: the chemicals background and the difficulty to ensure a representative exposure. In the field the tested substance can interfere with other substances used in the neighbourhood. This requires usually to move the treated hive to a non-agricultural area after the test, but this is artificial compared to the real conditions the field test is supposed to reproduce – this representative reproduction is the justification of the determining character of this test in the assessments. For the same reason, it is difficult to obtain the same conditions between treated fields and controls.

There is no easy way to measure bee exposure, even not through a pollen analysis. For this reason the field tests do not represent a sufficient reliability to be the highest tier tests in the assessment scheme when assessing substances detected in the foraged matrices.

Detection of contaminations through sowing dust

Dust contamination assessment is a general endpoint of PPPs assessment. Current measurements are based on dust gathered in Petri dishes put on a defined distance from the field side under the dominant winds. As a consequence of recent incidents in Germany, Italy and Slovenia, researches are carried out in order to define reliable methods to assess the dust spread. Honeybees and wild bees are likely to be contaminated by contact and through the morning dew harvest. The specific assessment of the risk for bees should take into account this specific exposure.

Synergies

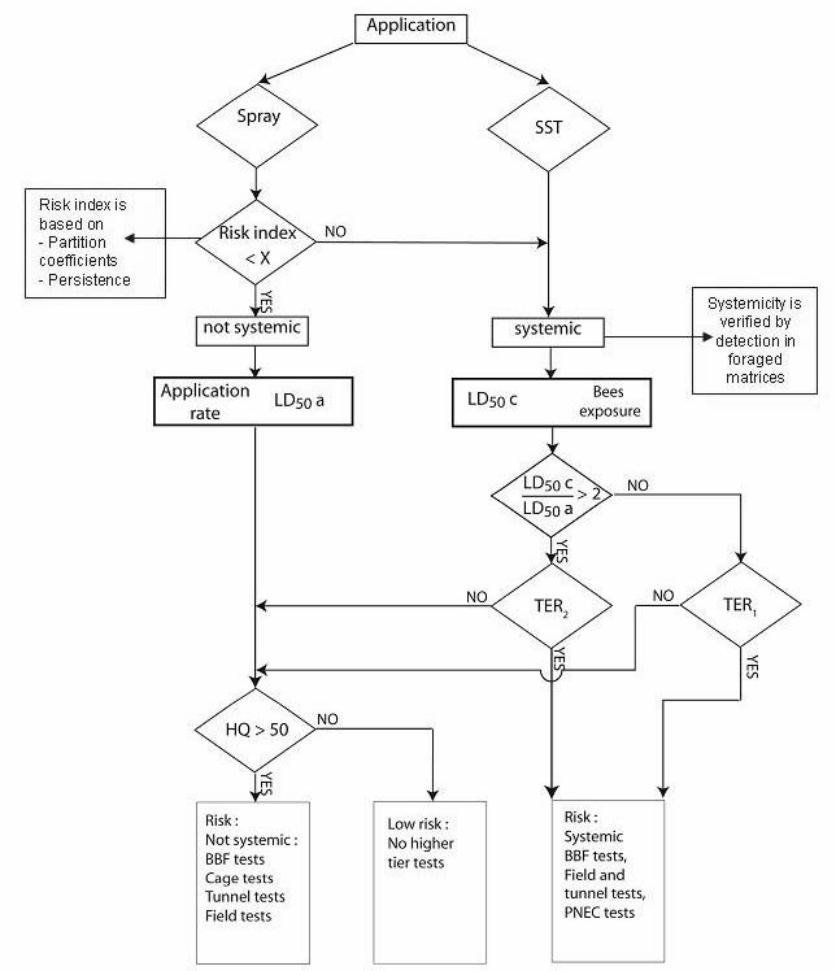
A same substance may have no effects at a certain dose when given alone, but may have a significant impact when associated with another substance. For instance, according to Vandame et al. (1998)[24], deltamethrin doses < 1,5ng/bee do not have effect on the thermoregulation of the bee, while an effect appears when deltamethrin is associated to prochloraz or difenoconazole. The association between insecticides and fungicides is frequently used. The legislation recommends testing the products in realistic conditions as far as possible. The synergies between products regularly associated in cultural practise must thus be tested. Seed coatings made of successive layers of different substances must be considered as a mix as the plant will absorb all these substances together. Synergies are likely to occur.

Reliability of studies

For all the tests we ask that reliability criteria would be established. The CST has established validity criteria for different types of studies (CST, 2004[8], pp. 40: dosage in the pollen and nectar, 51: chronic toxicity, 61: sub-lethal effects, 67: studies in cages,73: field tests).

A guideline should be established for the observation of behaviour in tunnels and fields. It should specify the length of observation time, the identified parameters, the counting method and any other means aimed at gathering objective information.

The quantities of pollen/nectar ingested by the different classes and castes of bees should be defined during an expert debate, on the basis of the 'the worse case' principle.



Conclusions

We propose a risk assessment scheme organised as follows:

- At the first tier, a risk index is calculated based on the persistence and the partition coefficient of the substance (as a indication for systemicity; pKow or pKoa is chosen according to the application mode, e.g. foliar or in soil or seed treatment).

- If the risk index shows a risk of systemicity, the presence of the substance is verified in the foraged matrices.
- If the substance is not present in the foraged matrices, the current assessment scheme is applied.
- If the substance is present in the foraged matrices, the acute mortality and the chronic mortality are measured and a TER is calculated.
- If the TER shows a risk, a complete assessment is performed (including bee brood feeding test and PEC/PNEC assays and calculation).

The process is summarized in the scheme below (see Figure).

Before implementation, further research is needed to determine:

- quantities of pollen consumed by the winter-bees,
- effects of small doses on the bee immune system when the substance is persistent.

The current risk assessment scheme for PPPs is not adapted to systemic substances, particularly those suspected to be present in pollen and nectar. It is important to take into account the persistence and systemicity of the substances, to put these parameters in relation with toxicity and with bee exposure, and to define methods capable of assessing the potential chronic lethal and sub-lethal effects. A fundamental change in the assessment scheme is critical for the survival of bees and other wild pollinators. We hope to have contributed to this reflection.

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III. Bumblebees and other bee species

The impact of different concentrations of a pyrethroid insecticide on the cyclic gas exchange cycles on bumble bees

*Karise, R., Kuusik, A., Mänd, M., Metspalu, L., Hiiesaar, K., Luik, A.

Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, 1 Kreutzwaldi St., 51014 Tartu, Estonia, *Reet Karise, PhD, reet.karise@emu.ee

Abstract

Minor effects of pesticides may remain unnoticeable in adult bees because of no visible changes in their behaviour throughout several days after coming into contact with pesticides. The hypothesis of this work is that changes which are not observable through the behaviour of the bumble bees can be seen through physiological patterns. The aim of the present research was to study the effect of low concentrations of Fastac 100 EC on discontinuous gas exchange cycles of bumble bee *Bombus terrestris* foragers. Using a system of flow-through CO_2 respirometry, the effect of different concentrations of alpha-cypermethrin on bumble bee foragers was studied. We found that the concentration of Fastac 100 EC that is used in the fields and a tenfold solution of that caused significant decrease in the frequency of bursts of CO_2 releases in bumble bees. 20-fold diluted solution did not cause the significant decrease. The lifespan of treated bumble bees also decreased by the field concentration and ten-fold diluted concentration. Alpha-cypermethrin caused changes in the respiration patterns of *B. terrestris* foragers although not always seen through the behaviour. These changes could potentially lead to a decreasing individual and colony survival.

Keywords: Respiration cycles, pyrethroid insecticide, bumble bees

A monitoring study confirming the safe use of DuPont Steward insecticide (a.s. indoxacarb) for natural bumblebee populations in flowering apple orchards and recommendations for the use of commercial bumble bee hives in flowering apple and pear orchards treated with Steward

Jozef J.M. van der Steen[1], Axel Dinter[2]

[1]PRI Biointeracties, Postbus 16, 6700 AA Wageningen, The Netherlands
[2]DuPont de Nemours (Deutschland) GmbH, DuPont Str. 1, 61352 Bad Homburg v.d.H., Germany

Abstract

In spring 2006 a monitoring field study was conducted to assess naturally occurring bumble bees in flowering apple orchards. Spread over the Netherlands, Belgium and Germany, 19 orchard sites were selected. The occurring pollinators (i.e. honey bees and bumble bee species) were determined during visual observations of 30 flowering trees per orchard, once before and after commercial treatment with Steward and in insecticide untreated orchards. Generally bumble bees were much less abundant than honey bees (about 1:10). No indications for decrease or disappearance of natural bumble bee populations due to Steward application in flowering orchards were found.

Commercial bumble bee hives (Biobest multi-hives) were set up in 20 apple and pear orchards in the Netherlands, Belgium and Germany in spring 2006. During the flowering period the bumble bee were exposed to commercial Steward applications in 18 orchards, while two were insecticide untreated. Three bumble bee hives per orchard were kept continuously open over the whole observation period or only for 4-day exposure/foraging period with exposure during the Steward application or with exposure starting 1, 2 or 3 days after the Steward application. Steward application caused on average 25% and 22% mortality of worker bumble bees in the colonies that were actively foraging during spraying and in the colonies that started foraging one day after Steward application, respectively. Mortality of worker bumble bees in the colonies opened two and three days after Steward application was statistically significantly lower. Colonies of all treatments developed from 50 to over 150-300 bumble bee workers during the study period and no effects on brood or the survival of queens were observed in any of the treatments.

Based on good agricultural practices, it is recommended to close commercial bumble bee hives during the day of Steward application and to keep the hives also closed the day after application to minimize acute worker bumble bee mortality.

Evaluation of side effects of commercial biological pesticides on the beneficial insect, *Bombus terrestris*

Veerle Mommaerts[1], Guido Sterk[2], Guy Smagghe[1,3]

[1] Laboratory of Cellular Genetics, Department of Biology, Faculty of Sciences, Free University of Brussels, Brussels, Belgium
[2] Biobest NV, Westerlo, Belgium
[3] Laboratory of Agrozoology, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Abstract

Nowadays cultivators are facing the problem that a percentage of their harvest is lost due to damage of pest insects or infections of plant pathogens. Meanwhile the use of pesticides is being limited because of environmental and residual risks and the development of resistance. Microbiological control agents (MCAs) are now widely used in integrated pest management (IPM) programs as an alternative for the conventional pesticides. MCAs include bacteria, yeast-like fungi, yeasts and viruses. In the field MCAs are dispersed in the crops by spraying applications. It is not unlikely that pollinators like bumblebees are exposed to these

biological pesticides while they are foraging. Biological pesticides may contain biological active materials that could grow on or in the insect. Therefore possible adverse effects on beneficial pollinators must be evaluated as pollination must be guaranteed.

This study has examined the potential adverse effects of commercial biological pesticides that contain bacteria, fungi, yeasts and viruses on the bumblebee *Bombus terrestris*. Worker bees were exposed under laboratory conditions to the maximum field recommended concentration (MFRC) of each compound via three different routes of exposure: dermal contact and oral feeding via the consumption of treated sugar water and pollen. In general all tested MCAs were found safe for workers of *B. terrestris*, with the exception of Botanigard *(Beauveria bassiana GHA*) via dermal contact treatment that caused 90% worker mortality at its MFRC after 12 weeks. Even at half of the MFRC, 50% mortality was observed, but there was no mortality with a lower dose of 1/10 of the MFRC.

Apart of to acute toxicity also sublethal effects on nest reproduction were examined. Here none of the tested compounds did exert detrimental effects as the production of drones after 12 weeks appeared to be not significantly different from the control nests (39.5±6.7) ($P>0.05$).

Overall, the results demonstrated that most of the biological pesticides tested can be considered as safe for *B. terrestris*, but some can be harmful. Therefore it is recommended that before any use in combination with pollinators all should be tested. In this context it is also advised that these compounds should be evaluated for potential effects on the foraging behavior in more field related tests.

Side effects of commercial *Bacillus thuringiensis* insecticides on micro-colonies of *Bombus terrestris*

Veerle Mommaerts[1], Guido Sterk[2], Guy Smagghe[1,3]

[1] Laboratory of Cellular Genetics, Department of Biology, Faculty of Sciences, Free University of Brussels, Brussels, Belgium
[2] Biobest NV, Westerlo, Belgium
[3] Laboratory of Agrozoology, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Abstract

Bacillus thuringiensis (*Bt*) is a natural soil bacterium that is used worldwide for the control of pest insects as its protein crystals possess insecticidal activity. Due to the intensive use of Bt in different crops like vegetables, ornamentals, flowers and fruiting plants, the question has raised whether Bt is safe for non-target organisms. Nowadays cultivators are using beside honeybees also bumblebees for the pollination of their crops such as tomatoes.

In this study the risk of two different strains of commercial Bt insecticides, *B. thuringiensis kurstaki* (Dipel® WG) and *B. thuringiensis aizawai* (Xentari® WG) on the biology of the bumblebee *Bombus terrestris* was assessed. In order to evaluate potential lethal and sublethal effects on the reproduction, micro-colonies of worker bumblebees were exposed to 0.1% of each compound, representing the maximum field recommended concentration (MFRC), and this via three different routes of exposure: dermal contact and oral feeding via treated sugar water and treated pollen.

For both Bt compounds no loss of survival was scored after dermal contact treatment. Via treated sugar water, Xentari® at 0.1% killed all worker bumblebees, but with a lower dose of 0.01% (1/10 of the MFRC) mortality was zero. With Dipel® at 0.1% in the sugar water and in the pollen, no mortality was scored.

Next to lethal effects, also sublethal effects were evaluated. In the nests exposed to Xentari® at 0.1% via the pollen a significantly lower number of drones was produced ($P<0.05$). However, no detrimental effects were seen with a lower dose of 0.01% ($P>0.05$). For the treatments with Dipel®, the reproduction in the micro-colonies was normal (37.6 ± 5.5 drones per nest) as in the controls (39.5 ± 6.7 drones per nest).

Then in a next step in our risk assessment study on side effects we evaluated the impact of sublethal concentrations of Xentari® (0.01% via the sugar water and the pollen) on the foraging behavior of bumblebees with a new experimental setup in the laboratory. Here no change in the behavior of the workers was seen.

Overall the results showed that the tested Bt insecticides cause an effect on the biology of *B. terrestris*. However, more information about relevant environmental concentrations is necessary before making final conclusions about the compatibility of these compounds with *B. terrestris*.

Can pesticide acute toxicity for bumblebees be derived from honeybee LD50 values?

J. van der Steen[1], L. Bortolotti[2], M.P. Chauzat[3]

[1]PRI Biointeracties, Postbus 16, 6700 AA Wageningen, The Netherlands, [2]CRA-API, Italy, [3]AFFSA, France

Abstract

Pesticide acute toxicity towards animals is commonly assessed using lethal doses (LD_{50}). The LD_{50} can be generated with two routes of exposure: when animals ingest the pesticide (oral LD_{50}) or when it is in contact with it (contact LD_{50}). Toxicity values for honeybees are usually used in ecotoxicological risk assessment infering that honeybees represent the pollinating insects. LD_{50} values are also measured for bumble bees but to a lesser extend.

The first step of this exercise was to collect known LD_{50} (contact and oral) values measured for both honey bees and bumble bees.

Based on the LD_{50} values of 20 pesticides, the relationship between oral LD_{50} values of honey bees and bumble bees was calculated with the regression formula. The same calculation was done with contact LD_{50}. Results showed that there was an approximate relationship; toxic active ingredients for honey bees were also toxic for bumble bees. However, when honey bee LD_{50} values in the toxic range (LD_{50} < 1 µg/bee) and less toxic range (LD_{50} > 1 µg/bee), were compared to bumble bee LD_{50}, the relationship was very much less statistically significant. This both counted for the oral and contact LD_{50} values. It is concluded that the known LD_{50} values of honey bees could indicate broadly a range of LD_{50} values for bumble bees. However, for toxic and less toxic substances, the LD_{50} for bumble bees cannot be derived from known honey bee LD_{50} values. It must be noticed furthermore that the LD_{50} values for honey bees, presented in literature and databases of universities and legislation offices vary significantly.

IV. Test methodology (laboratory, cage, field, sub-lethal, etc.)

Influence of the brood rearing temperature on honey bee development and susceptibility to intoxication by pesticides

Piotr Medrzycki, Fabio Sgolastra, Laura Bortolotti, Simone Tosi, Gherardo Bogo, Erica Padovani, Claudio Porrini, Anna Gloria Sabatini

Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Unità di Ricerca di Apicoltura e Bachicoltura, Via di Saliceto 80, 40128 Bologna, Italy

Abstract

The brood rearing temperature is one of the most precisely controlled physiological parameters in a honey bee colony. Adult bees keep the brood area centre at 35 ± 1°C. In order to maintain the temperature within this narrow range, the high or low external temperature is contrasted by thermoregulation behaviours. Thus, normally only slight deviations from the optimal level may occur. Nevertheless, in particular situations the brood may be subject to conditions of suboptimal temperature. For example, a slight bee poisoning, causing

the loss of apparently insignificant quantity of adult bees in early spring, i.e. in the conditions of low external temperatures, could impede to maintain the brood at the constant optimal temperature. It was hypothesised that the honey bees deriving from the brood kept at suboptimal temperature might be characterised by lower fitness and by higher susceptibility to pesticide intoxication. This could lead to consistent adult bee losses delayed in time. In previous studies, adult bees, reared at suboptimal temperature during pupal development, showed decrement in short-term learning and memory capacities. These bees could have difficulties to carry out thermoregulation behaviour causing, again, reduced brood rearing temperature.

The present study was aimed to investigate if the decrease of the brood rearing temperature of only 2°C may have effects on the larval mortality and on the adult emergence and life parameters. Moreover the susceptibility to the intoxication by pesticides was studied both on the larvae and on the adults emerged from the brood reared at the tested temperatures. For this purpose, lab trials were conducted basing on Aupinel's protocol for the *in vitro* rearing of honeybee larvae. The larvae were exposed to two temperatures: 35°C (optimal) and 33°C (suboptimal) from 12h after hatching until 15 days of age. According to the experiment, dimethoate was administered either to larvae or to adults. Larval mortality, adult emergence and longevity were measured. The mortality both of the larvae and of the adults after the dimethoate administration was also recorded.

Our results showed that the lower rearing temperature has no negative influence on the larval susceptibility to the intoxication with dimethoate. The LD50 (48h and 72h) was even higher for the larvae reared at lower temperature than for those reared at the optimal temperature. The adult emergence doesn't seem to be influenced by the rearing temperature, but the longevity is strongly reduced in the bees deriving from the cool-reared brood. The mortality rate of adults emerged from larvae reared at the suboptimal temperature is comparable to that of adults intoxicated with the LD50 of dimethoate emerged from larvae reared at the optimal temperature. Thus the low-temperature-brood-rearing seems to be an important stressing factors with the effects on the adults.

Field testing methodology for investigating the effect of systemic insecticides on honey bees

I. Tornier, (Eurofins Agroscience Services), M. Coulson (Syngenta), S. Hecht-Rost (Eurofins Agroscience Services), N. Ruddle (Syngenta)

Abstract

Elsewhere at this symposium risk assessment schemes are being proposed for systemic insecticides. The purpose of this presentation is to demonstrate methodologies already used for systemic seed treatment insecticides. Investigations involved two main designs:

- semi field (tunnel) trials, assessing residues in plants, pollen, and various hive products;
- open field studies investigation the long term developments of honey bee colonies. Colnies were followed for a long time period, including overwintering. Parameters studied included: mortality, foraging activity, brood development, hive weights, disease analysis (e.g. *Nosema apis*, *Varroa destructor*, American foulbrood, bee viruses).

Behavior of honey bees; a guideline to assess troubles in bee foraging activity under insect-proof tunnels

Hervé Giffard, Olivier Mamet

Testapi, station d'expérimentations apicoles, 464, Sarré, 49350 Gennes
Francegiffard.testapi@wanadoo.fr, tel.+33 241 526 894, fax. +33 241 526 208

Keywords: bee behavior, bee presence, chronic effects, cleaning behavior, paralysis, seed treatment

Introduction

The usual guidelines [1,2] for honey bee risk assessment have been validated for acute effects to honeybees following foliar applications of agro pharmaceuticals during flowering. However the use of coated seeds and soil treatments during the sowing operation are being suspected to induce chronic effects on the bee foraging activity during the time of flowering. These effects had not been investigated before the methodology described below was developed.

This new method addresses chronic effects that can be observed in fields where honeybees forage sunflowers grown from insecticide coated seeds. It does not deal with the acute effects of such chemicals but only aims at identifying any troubles that could be caused by residues remaining in the plant at the time of flowering.

Method development

Inventory of parameters

Apidologists and French beekeepers have listed a series of 'troubles' in bee foraging activity [3, 4]. All of them agree that there are signs of decline of bee colonies. These signs had to be listed and assessed to find out which belong to normal behavior and which not: these are considered as 'troubles'. These troubles would not cause mortality at the short term, but might cause a decline on the long range.

The list of troubles from beekeepers was very long with many parameters difficult to record. First of all it was necessary to define 'normal foraging activity', in order to be sure that other signs could be recorded as 'trouble' in the foraging activity. We selected few parameters easy to observe by technicians, in order to provide reliable data.

Parameters to be observed

The number of forager bees working normally is previously counted. Observed troubles usually affect a few individuals only. Parameters to be observed refer to 3 different levels: presence signs, cleaning signs and clinical intoxication signs.

Presence signs: This parameter refers mainly to motionless bees on the flower and to bees on the whole plant but not on the flower, with agreed definitions of a moving bee and a motionless bee.

Cleaning signs: The staff observes and counts the bees that clean themselves in two ways: (a) limited cleaning of legs and horns (as flies and butterflies do), (b) overall cleaning (the whole body is brushed with middle or hind legs). These observations should be made for at least a few seconds and sometimes for several minutes for one bee.

Clinical intoxication signs: These are at the highest level on the 'trouble' scale. Hanging bees are specially observed. Bees hang from leaves or from flowers by one or two legs. Sometimes bees are motionless, sometimes they clean themselves. Any such honey bee is supposed to fly away when pushed by the technician's finger and is counted as 'hanging bee'. In fact the bee often falls and lays down and is counted as a 'falling bee', which seems a more important trouble. The last kind of sign is close to acute effects with paralysis and disordered wings or legs.

Recording of signs: All parameters are defined beforehand in recording booklets. The staff needs to be trained before the trial for a unequivocal interpretation of observations. The observations are recorded daily in booklets during the whole flowering period. They provide raw data used to compare the treatments. Raw data are used to build up boards and graphs in order to detect potential troubles. However, not all signs observed are 'troubles'. First trials in 2003 and 2004 showed expected troubles in the control too. Bees clean themselves and some others die daily in all bee colonies. It is the frequency and the number of signs within different modalities which makes the difference.

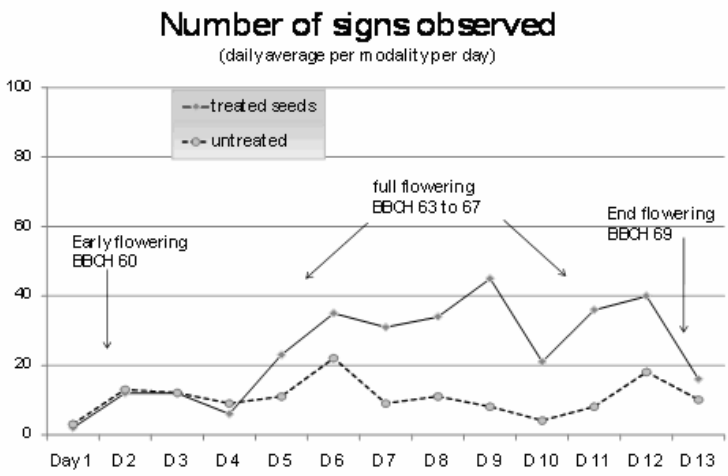
Trial design

The further trial design is the same as for acute toxicity test under insectproof tunnels. There are two modalities, a control (untreated) and a test item (treated). Treatments are use of coated seeds, or sowing operation with a granulator. Replication of these modalities for more consistent data are possible. A toxic reference is neither necessary nor recommended because potential effects are merely compared to a normal activity, the agrochemical industry would not agree to have a seed treatment as a toxic reference, and the use of soil treatments or coated seed treatment are not compared to a worst case. Sunflower appeared to most suitable crop for such observations. Bees have a large place to land on sunflowers, and stay quite a long time foraging nectar and pollen therefore they are easy to count and to observe.

Also regarding equipment the design is similar to acute toxicity tests under insect-proof tunnels of 140 m² each. These tunnels can contain real small colonies. The assessments of daily mortality and quantitative foraging activity are completed with observations of qualitative foraging activity. Usually it is important to prove that there are neither acute effects nor differences in mortality between the modalities.

Results and discussion

Such trials have been conducted with several products and crops over the past five years. Registered data appeared adequate for statistical analysis. The number of observed troubles was usually not very high compared to hundreds of forager bees. When a specific parameter such as 'presence sign' gave a very limited number of data in both modalities, it was necessary to cumulate the results of several different signs in order to get sufficient data. In this way the difference can appear to be significant or at least give information on the predominance of certain troubles during the period of high bee activity.



In early flowering as well as in end of flowering the number of observed troubles was not sufficient and differences could be not significant. On the contrary, from early flowering to full flowering the increased number of troubles provided consistent data. The difference in the number of troubles in foraging activity was significant between modalities.

When honeybees forage a tunnel of a limited surface (about 140 m²) for 10 to 15 days, potential effects or troubles can be observed. Extrapolation of such results would therefore suggest a risk of more important troubles when forager bees visit hundreds of hectares during 1 to 2 months.

Conclusion

This methodology was developed as a tool and a guideline in the risk assessment scheme for honey bees. It is now recommended in France[5] to assess potential troubles of all kinds of coated seed treatments and soil treatments on sunflowers.

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A methodology to assess the impact on bees of dust from coated seeds

Hervé Giffard[1], Thierry Dupont[2]

[1]Testapi, Station d'expérimentations apicoles, 464, Sarré, 49350 Gennes, France, giffard.testapi@wanadoo.fr
[2]Qualilab, 1121 rue de la Bergeresse, 45160 Olivet, France

Introduction

During springtime of 2000 to 2003 much bee mortality were observed in France when sowing maize and sunflowers.

During 3-4 years beekeepers claim high mortality rates in their apiaries at the time of sowing maize and sunflowers, mainly during April and May. Blossoming crops or bad agricultural practices were not suspected (as there was neither rape seed crops nor other blossoming crops at this time), but only wild plants such as dandelion or flowering trees in the field hedges.

After several meetings with the Agricultural authorities in the South West of France and a review of different hypothesis, it was decided to investigate on dust seed being disseminated when sowing. As coated seeds were mainly used in this area, there was a suspicion of a possible contamination due to dust produced by coated seeds.

By chronological correlation seed dusts from insecticide coated seeds were finally suspected to induce these mortalities.

After a review of different coated cultivars sown in closed conditions it was decided to assess the effects of two modalities in agricultural and laboratory conditions.

The question was: 'Is there a possibility that insecticide dust be disseminated during sowing and contaminate wild flowers that are being foraged by honeybees?'

Experimental methods

First indoor tests were conducted with non moving sowing machines equipped with paper filters in order to catch dust that is disseminated in the air while the engine is running. Different kinds of coated seeds were then tested. Some seed released dust while others did not.

Testapi was requested and incited to carry out a test in order to investigate potential effects on honeybees of plants exposed to dust released during sunflower sowing.

A study extended to laboratory to assess potential effects on honeybees was conducted outdoors, simulating sowing of treated seeds. Assessments were conducted under controlled conditions to monitor bee exposition to foliage in small containers, similar to LD50 tests. This methodology is based on the reference of EPA guideline relative to residues on foliage.

Following previous dustiness test, two sunflowers seed varieties were chosen with insecticide coated seed treatment (Melody and LG 5660).

Two fields distant about 3 km were selected. The surface sown was 2.2 ha in each field. Application procedures were identical with cleaning of the pneumatic applicator of 4 sowing rows in both fields.

The plant species used as the receiving target of dust was *Tibouchina*, an ornamental species known for its hairy leaves that represents a worst case for this purpose, as pile on leaves facilitates dust retention.

The test design had 4 treatment groups: the two varieties, a control and a toxic standard. Application of toxic standard was done in an open space close to the laboratory.

Plants were placed in fields before the sowing started and remained in the fields for 2 days. The control group received no treatment. The toxic reference was treated with a liquid spray of dimethoate, in order to ascertain bee sensitivity.

Two tests were carried out, first with bees introduced in containers with foliage collected 2 hours after sowing and then with new bees introduced in new containers with foliage collected 24 hours after sowing.

Bees were taken from one sole and healthy beehive and distributed in the 4 groups and containers at random.

The surface in each container was covered with foliage taken from plants. The surface of foliage in the container was adapted with scissors to be exactly similar in cm². Then 20 honeybees were introduced in all boxes to be in contact with *Tibouchina* leaves. The foliage was removed after 24 hours but bees were kept in boxes for 2 more days. This made the duration of the test 72 hours.

The containers were placed in controlled conditions of about 26°C in temperature and over 60% relative humidity, there bees were fed with a safe sugar solution.

Remind that we had 4 treatment groups with 3 replicates of 20 bees in each group that makes 60 bees per group and 240 bees for each of the 2 tests. Mortality assessments were made at 4 hours, 24, 48 and 72 hours following exposure.

Results

From the raw data we calculated the average mortality in the 3 replicates of each treatment group using usual formulas in statistical analysis. These results were validated by mortality rates at 24 hours of 0% in the control and over 90% in the toxic standard.

The results on the two sunflower varieties are important as a validation of the use of this new study protocol.

	Average Bee Mortality in %							
	First test				Second test			
	4 h	24 h	48 h	72 h	4 h	24 h	48 h	72 h
Control	0	0	5	17	0	0	2	8
Toxic standard	31	100	100	100	12	94	94	98
Sunflower Melody	0	3	25	40	0	0	7	22
Sunflower LG 5660	0	2	5	8	0	0	0	5

Discussion and conclusion.

With no cross contamination possible, some lethal effects on bees were observed following the use of one treated seed and absolutely no effect for the other one. Experimental conditions were satisfactory as there was no wind at all and dust lay down around in the field. A little wind could have blown away the dust into hazardous directions. To ensure a better exposure it will be necessary to sow maize or sunflower insecticide coated seeds around plants placed in the middle of the field.

Following this first study, French authorities set up a 'dust schedule' to seed coating factories limiting the dust discharge to 4 grams per quintal (100 kg) of coated seed which corresponds to the safe variety (LG 5660) in above described test.

Since 2004 no more high mortalities have been attributed to sowing operations in France. This results should be of high interest for other European countries. This methodology should therefore have a place as a guideline in the regulation scheme in European countries.

Sublethal effects of fipronil on the ability of honeybees (*Apis mellifera* L.) to orientate in a complex maze

Axel Decourtye[1]* , Samuel Lefort[1], James Devillers[2], Monique Gauthier[3], Pierrick Aupinel[4], Michel Tisseur[1]

[1]ACTA, ICB-ENVL, 1 avenue C. Bourgelat, 68280 Marcy l'Etoile, France*
[2]CTIS, 2 chemin de la gravière, 69140 Rillieux La Pape, France
[3]CNRS, Centre de Recherches sur la Cognition Animale, Université Paul Sabatier, 118 Route de Narbonne, 31062 Toulouse cedex, France
[4]INRA, Unité expérimentale d'entomologie Le Magneraud, 17700 Surgères, France
*Tel.: 334 788 756 22, Fax: 334 788 756 20, E-mail: axel.decourtye@acta.asso.fr

Abstract

Background: The recent fipronil-based pesticide is accused by bee-keepers of causing depopulations in hives of honeybees (*Apis mellifera* L.). Behavioural effects during the flight of foraging honeybees would have been evoked. To test whether the insecticide fipronil may disorientate foragers, its impact on orientation in a maze was examined. Bees had to fly through a sequence of boxes to reach the target, which was a feeder containing a reward of sugar solution. After being trained to associate a green mark with the reward, foragers received 1 µg kg^{-1} fipronil orally and their capacity to orientate through the maze following the colour mark was tested and compared to control.

Results: The rate of foragers entering the maze, and so responding to the mark placed at the entrance, was reduced with fipronil-fed animals. Before and after treatment, 86-89% of bees equally flew through the whole path and arrived to the goal without mistakes. The rate of fipronil-treated bees finding path without mistakes decreased to 60%. Conversely, the rate of bees with unsuccessful searches for the goal notably increased with treatment (34% in treated bees *versus* 4% in control bees).

Conclusion: Our results show that orientation capacities of foragers in a complex maze were affected by fipronil.

Keywords: *Apis mellifera* L., pesticide, maze, conditioning, visual learning, flight

Introduction

Honeybees can accurately and repeatedly navigate to a food source, and then communicate to their nest mates the distance and direction to reach it.[1] The process of foraging involves learning and memory, communication, navigation, taking into account information from the internal clock and many other flexible responses, e.g. the ability to integrate local landmarks.[2] These biological functions are potentially affected by pesticides. This is particularly true for the visual learning of landmarks which is important in spatial orientation.[3,4] One of the major tasks for the honeybee during a foraging flight is to learn and recall many complex visual patterns.[5] It is well known that honeybees use landmark-based cues to navigate to a goal and to return to the nest. These cues are needed to set the flight direction, to monitor progress to the goal, to provide intermediate guiding landmarks and they finally aid in spatial tracking the target when the bee is in its vicinity.[3] Considering the neurobiological functions in orientation processes, it is of great interest to know whether neurotoxic insecticides induce behavioural disturbances and if these alterations exist at low concentration level. It is now well-admitted that sublethal concentrations of pesticides can affect the spatial orientation of the honeybee.[6] In an insect-proof tunnel (feeder located at 8 m from the hive), Vandame et al. (1995) showed that deltamethrin altered the homing flight of foragers treated topically at sublethal doses.[7]

Accordingly, when insecticide intoxication is suspected, bee losses observed in field conditions could be attributed to alteration of the flight pattern between a contaminated food source and the hive. More significantly, the impairment of homing flight of exposed foragers is a possible cause in the Colony Collapse Disorder. This syndrome was principally found in North America and Europe, where beekeepers have recently claimed to observe a complete absence of adult bees in colonies, with little or no build-up of dead bees in or around the colonies.[8-11] In recent years, French beekeepers reported that hives located near sunflowers, originated from seeds dressed with Gaucho® or Régent TS®, show high levels of damage due to a progressive decline in the hive populations, until a complete loss of the colonies.[12] The imidacloprid- and fipronil-based products are accused by French bee-keepers of causing behavioural effects in foragers and subsequently no homing return to hive. So, many studies were carried out in order to assess the effects of these insecticides on behavioural traits, and more particularly those involving in the foraging. Using conditioning of the proboscis extension reflex in restrained individuals, previous studies showed that fipronil in acute topical application or chronic ingestion impaired olfactory learning of bees.[13,14] But, it is not clear whether the endpoints tested in these sublethal studies can be clearly related to the respective field effect of concern.[15,16] In contrast, the ecological relevance is better in the methods on orientation and homing ability.[6,7,17,18]

To test whether fipronil may disorientate foragers, its impact was examined on orientation of honeybees in a maze under outdoor conditions. Orientation performance of bees in a complex maze relies on associative learning between a visual mark and a reward of sugar solution.[19] We studied whether foragers receiving orally 1 µg kg^{-1} of fipronil can orientate themselves through the maze.

Materials and Methods

Insects

Experiments were repeated twice, each time with a colony of honeybees (*Apis mellifera* L.) of about 20,000 workers and a fertile 1-year-old queen. Honeybees were confined in a 5-comb hive (2 brood combs, 2 honeycombs and one empty comb). The colony was maintained in an outdoor flight cage (2.5 m × 2.5 m, 2 m high) covered with an insect-proof cloth (2 mm × 2 mm mesh) and a ground covered with a plastic. Any dead bees found on the ground were counted and discarded daily.

Feeding

A feeder was positioned about 1.5 m from the hive entrance, filled with sucrose solution (500 g kg^{-1}) and multi-floral pollen. The sucrose solution was delivered in a dish, 7 cm in diameter, made of a material impervious to ultraviolet rays.

Device of the maze

The maze consisted of a matrix of 4 × 5 identical cubic boxes, each side of 30 cm, with each wall carrying a 4-cm diameter hole in its centre where bees crossed.[19] The maze was placed inside the flight cage on a table, 60 cm height. The boxes were made of white opaque Plexiglas and a metallic grate covered the maze (3 mm × 3 mm mesh).

Principle of the maze

Bees had to fly through a sequence of boxes to reach the goal – a feeder containing a reward of sugar solution. A path through the maze spanned 9 boxes, including 3 decision boxes and 6 non-decision boxes. A non-decision box had two holes, each in a different wall, where the bee entered through one hole and was expected to leave through the other tagged with a green mark. A decision box had three holes, each in a different wall, where the bee entered through one hole and then was expected to choose between two other holes: one with a green mark representing the correct path and another without mark representing the incorrect path which ultimately led to a dead end. Finally, the bee was released from the box in which she was trapped.

Conditioning procedure

During conditioning, bees were collectively taught to associate the mark with a feeder. For that, a green mark was fixed in front of a sucrose solution feeder outside the maze near the entrance during one hour. One additional hour, the feeder was placed in the first box of the path for about one hour, in the second box of the path the next hour, in the third box during one other hour and so on. Then, the feeder was moved on the fifth box during the same time.Finally, the feeder was placed at the end of the path (Fig. 1), in the reward box (9 cm × 9 cm), where all bees that underwent the conditioning procedure were individually marked with colour number tags.

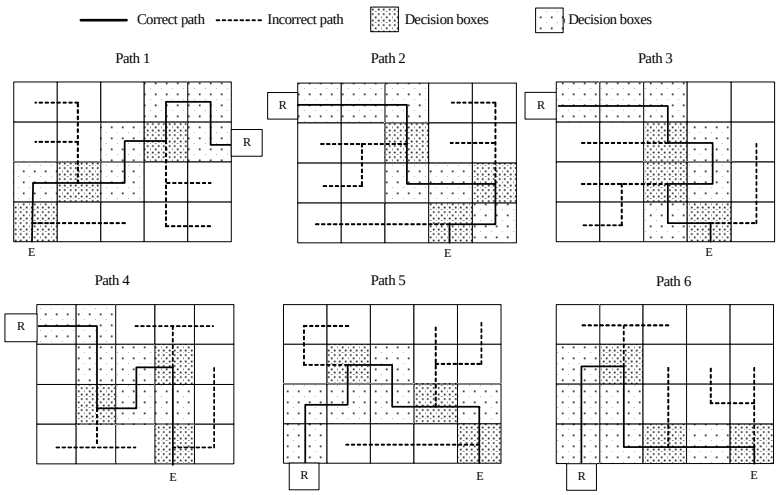

Figure 1 Maze paths used before, during and after treatment. Path 1 was used for the conditioning procedure and other paths were used for the retrieval tests. Each path started with the entrance (E), contained 3 decision boxes, 6 no-decision boxes, and finished with the reward box (R).

After the bee had found the goal, received her reward and was marked, she was released from the reward box and allowed to return directly to the hive (without flying back through the maze). A total of 185 bees were labelled during the untreated periods (100 before treatment: 58 for colony 1 + 42 for colony 2; 85 after treatment: 40 for colony 1 + 45 for colony 2) and 131 bees during treated periods (71 for colony 1 + 60 for colony 2). One-day conditioning period was necessary to train a sufficient number of bees. Each bee was trained only once.

Retrieval tests

After conditioning, the capacity of an individual bee to negotiate a path into the maze was tested. Green marks were affixed below the appropriate hole in each box to indicate the correct path. When a bee entered the maze, an observer noted the number and the colour of the tag, correct decisions, incorrect decisions and turns back. During retrieval tests, five different paths lasting 15-20 min were used (Fig. 1). Successive paths were interspersed with a cleaning containing ethanol to remove possible olfactory cues. During a test, only one bee was allowed into the maze at the same time and she was tested for one of the five path configurations. Bees were tested between 24 h and 32 h after training.

At the end of each day, a path was carried out without green mark inside the maze (only one mark stayed at the entrance). Therefore, any bee arrived at the goal within 5 minutes. This test confirms that green marks were the only internal landmarks used by bees as navigation cues.

Three-stage periods

We compared responses of honeybees before and after exposure to the insecticide on the same colony. Thus, performances of the honeybees were compared under various feeding conditions: sucrose solution without pesticide, with 10 ml l^{-1} ethanol (before and after treatment) and sucrose solution added with fipronil, with 10 ml l^{-1} ethanol (treatment). For each condition (controlled and treated), bees were submitted to conditioning and retrieval tests. Data of each period were obtained from different bees.

Oral treatment

Technical grade fipronil (98% purity, CAS RN 120068-37-3), purchased from Cluzeau Info Labo (France), was dissolved in ethanol (95-96% purity) and stock solution was diluted to final concentration in sucrose solution. The final concentration of ethanol was 10 ml l^{-1}. As a control, the sucrose solution was analysed (GIRPA, France) for contamination with HPLC/MS technique (limit of quantification = 0.5 µg kg^{-1}) to detect fipronil and its two mains metabolites (MB46136, MB46513). According to these analyses, the sucrose solution contained 1 µg kg^{-1} fipronil and was free of metabolites.

During the treatment period, fipronil was administered at the end of the conditioning period and before the test, then the honeybees consumed the contaminated syrup between 24 h and 48 h after training. The contaminated sucrose solution (1.2 litre) was delivered in a feeder placed outside the maze, and all the syrup was collected by foragers. During control periods (before and after treatment) honeybees were fed after training with a sucrose solution containing 10 ml l^{-1} ethanol.

Performance analysis

For each period, the performance of labelled bees, which entered the maze for the first time, was analysed. Four categories of performances were defined and a note was assigned to each of them:

- Bee flows through the path and arrives directly to the goal (reward box);
- Bee flows through the path and arrives to the goal with one or more turns back (bee leaves the box through the hole from which it entered);
- Bee flows through the path with mistake(s) (bee making one or more wrong turns at the decision boxes) but arrives to the goal;
- Bee does not arrive to the goal within 5 min after entering the maze.

- Each bee received a note corresponding to her performance. Performances of control and fipronil-treated bees were evaluated as the mean of notes assigned to bees in each group.

Flight time

The time required to reach the goal from the instant of entering the maze was measured for each bee. Flight time was considered only for bees flying through the whole path within 5 min. Honeybees that did not reach the goal within 5 min were excluded from this analysis.

Statistical analysis

A multifactor ANOVA (Type III sums of squares) was used: the dependent factors were number of dead bees, performance notes or flight times, and the independent factors were colonies, feeding periods (i.e., before, during of after treatment) or paths. We also checked for first-order interactions between the independent factors. For these statistical analyses, the data were log-transformed to achieve normal distribution.[20] Tukey's Honest Significant Difference test (THSD test) was performed on all analyses to assess pairwise differences between the feeding periods. Each comparison was carried out according to the Dunn-Sidak method,[20] at a critical probability of $\alpha' = 1 - (1 - \alpha)^{1/k}$, where k is the number of intended tests ($\alpha' = 0.0125$). To improve the illustration of performances and the comparability with other studies, we give in the text the percentage of bees ranked in the four performance categories according to the feeding period.

Results

Mortality

No significant differences were found between the two colonies and the three feeding periods (Table 1). The treatment with fipronil did not lead to additional mortality. The pooled number of dead worker bees for the two colonies was 2611 and 1934 for control periods (before and after treatment respectively) and 1982 for treatment period. Therefore, feeding honeybees with sucrose solution added with fipronil 1 µg kg^{-1} could be considered as a sublethal concentration.

Table 1 Effects of independent factors on mortality of honeybees (*Apis mellifera* L.).

n = 30[a]	**d.f.**	**Mean square**	**F value**	**p value**
Main effects				
Colony	1	0.00	0.21	0.648
Feeding period	2	0.04	1.86	0.177
Error	24	0.02		
Interactions				
Colony × feeding period	2	0.03	1.65	0.213

Results of multi-way ANOVA with first-order interactions are given. [a] Number of days where mortality was recorded.

Performance

Data collected from the two colonies and the five paths were pooled in Fig. 2 to show the percentages of bees assigned to each performance category during retrieval paths tests. Control and fipronil-treated bees made no mistakes and consequently category 3 is empty. Before and after treatment, a high percentage of bees flew through the path and arrived directly to the goal (category 1: from 87 to 89%). In the same time, a low percentage of bees made turns back (category 2: from 6 to 9%) or failed in reaching the goal (category 4: from 4 to 5%). Thus, bees without treatment trained to follow colour marks were able to use the same cue to find a new way in a path they had never encountered previously. The rate of fipronil-treated bees reaching the goal directly decreased to 60%. In parallel the rate of bees that did not reach the goal within 5 min notably increased to 35%. In this group, foragers stopped during the trip, remaining in a box and flying inside. The number of turns back (category 2) was not different between control and treated-bees. The number of fipronil-fed foragers entering the maze, and so responding to the mark placed at its entrance, was

reduced. Only 15% of labelled bees were observed into the maze during the treatment period, compared to 34% and 41% before and after treatment, respectively.

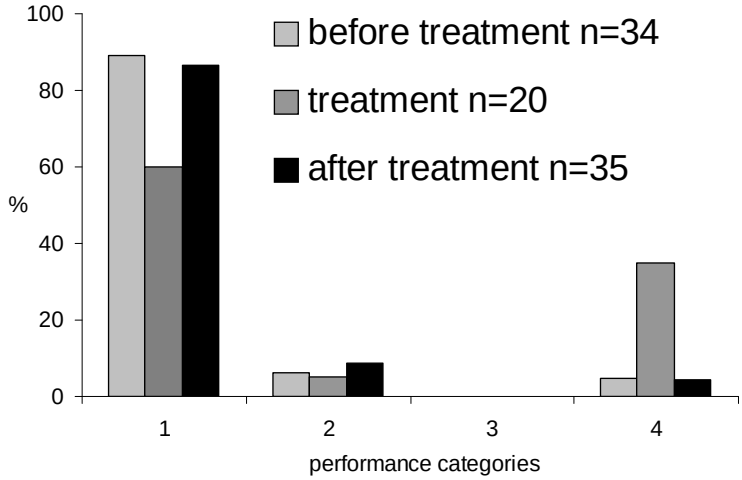

Figure 2 Performance of bees ranked into 4 categories.

Performance analysis with three-way ANOVA showed no significant differences between paths, whereas feeding periods significantly differed (Table 2). This difference was nearly statistically significant between colonies. Honeybees' performance before and after treatment was not significantly different (THSD tests; $p = 0.35$; Table 3). Bees orally exposed to fipronil had significantly lower performances than untreated bees (THSD test; before treatment: $p < 0.001$; after treatment $p < 0.01$). There was significant interaction effect between colony and path (Table 2). But in separate analyses, the performance of both colony 1 ($F = 3.46$, $p = 0.072$) and colony 2 ($F = 2.31$, $p = 0.069$) did not differ significantly between paths.

Table 2 Effects of independent factors on performances of foraging honeybees (*Apis mellifera* L.).

n = 89[a]	**d.f.**	**Mean square**	**F value**	**p value**
Main effects				
Colony	1	0.11	3.69	0.057
Feeding period	2	0.22	7.07	0.001
Path	4	0.02	0.61	0.654
Error	120	0.03		
Interactions				
Colony × feeding period	2	0.02	0.69	0.505
Colony × path	4	0.08	2.89	0.025
Feeding period × path	8	0.01	0.53	0.843

Results of multi-way ANOVA with first-order interactions are given. [a] Number of bees taken into account for performance evaluation.

Flight time

In bees ranked in categories 1 and 2 the time required to reach the goal from the instant of entering the maze was measured. Flight time of forager bees did not differ significantly between colonies and path but differed between feeding periods (Table 4). On average, before and after treatment, bees flied through the maze in 59 s and 40 s, respectively (Table 3). Fipronil induced a significant increase of bees' flight time through the maze ($p < 0.01$). The mean duration of the flight was of 93 s. Thus, the bees' ability to negotiate the maze following a colour mark was reduced by treatment.

Table 3 Performance notes and flight times from honeybees (*Apis mellifera* L.) to three feeding periods.

	Performance notes	Number of bees	Flight times (s)	Number of bees
Before treatment	1.20 ± 0.08 (a)	34	59.48 ± 6.42 (ab)	32
Treatment	2.10 ± 0.32 (b)	20	93.15 ± 20.03 (b)	13
After treatment	1.22 ± 0.10 (a)	35	40.40 ± 4.44 (a)	33

Mean ± s.e.m. and number of bees are given. Letters indicate significant differences (THSD test; $p < 0.01$).

Table 4 Effects of independent factors on flight time of foraging honeybees (*Apis mellifera* L.).

n = 78[c]	d.f.	Mean square	F value	p value
Main effects				
Colony	1	0.01	0.10	0.756
Feeding period	2	0.47	5.78	0.004
Path	4	0.08	1.03	0.397
Error	108	0.08		
Interactions				
Colony × feeding period	2	0.14	1.74	0.180
Colony × path	4	0.13	1.60	0.179
Feeding period × path	8	0.02	0.60	0.873

Results of multi-way ANOVA with first-order interactions are given. [a] Number of bees taken into account for flight time evaluation.

Discussion

Our experiments show that honeybees, in flying situation, can associate a visual mark to a reward, a result already observed by Menzel et al. (1974) [21], and they can use this associative learning to negotiate a path in a complex maze.[19] The retrieval tests point out the capacities of bees to restore the rule previously learned that the colour predicts the location of food. After treatment with 1 µg kg^{-1} of fipronil, the ability of bees to perform the task was impaired compared to control bees. The significant features for intoxication are the small number of honeybees entering the maze for the test, the relatively poor rate of honeybees reaching the goal directly with an increasing flight time and the increased rate of honeybees that did not find the goal during the 5-min observation period. Control bees can successfully locate the goal (sugar solution) by flying through paths they have never previously encountered, but this task was more difficult for treated bees. Treated bees that displayed unsuccessful searches for goal remained and flew into a box, without using the local landmarks to reach the goal. They landed on the grid, towards the light and this behaviour probably indicates a modification of phototropism. The fact that insecticide-treated bees fly in the sun direction was previously shown by Vandame et al. (1995).[7] But, fipronil-treated bees made no more errors and turns back than control bees.

It is possible to divide the fipronil-treated honeybees into three categories: those previously conditioned but which do not come back to the maze for testing, those recorded during testing but which are lost in the maze and those which succeed taking more time to reach the goal. How explain these different reactions to treatment? This complex panel of behavioural modifications we have observed can be linked to different levels of intoxication related to the dose ingested by each bee. According to the model of exposure previously developed by Rortais et al. (2005),[10] a nectar forager would have ingested between 0.03 and 0.11 ng of fipronil in our experimental conditions. These doses are inferior to the median lethal dose value of

fipronil (LD50 determined 48 h after the oral treatments was 6 ng per bee in our laboratory[14]), confirming the sublethal character of treatment.

As no extra mortality was associated to fipronil treatment, we can suppose that fipronil decreased the motivation of honeybees to come back to the maze. Fipronil ingested after the training period should be perceived as a repulsive agent, foragers could associate the green mark to a negative reward and avoided it during the retrieval test. These effects are classically attributed to an anti-feeding character of the compound.[22,23] But a decrease of foraging activity can also be due to processes occurring inside the hive. For example, Kirchner (1999) reported a reduction in the foraging activity on a food source contaminated with imidacloprid (20-100 µg kg^{-1}) due to the induction of trembling dances that prevent other bees from foraging.[24] In addition, a lower motivation to perform waggle dances revealed a reduction in the recruitment activity. Thus, the changes in the communication process can also result in a decreased foraging activity.

The mean flight time in the maze ranged from 40 s to 59 s in untreated bees, and reached 96 s in fipronil-treated bees. The impact of fipronil on the flight-time would not be surprising because fipronil's main targets, the receptors to the neurotransmitter γ-aminobutyric acid (GABA) located on the membrane of the muscle cells, play an important role in modulating locomotor and flight activity in insects.[25,26,27] Fipronil may act at the peripheral neuromuscular junction of muscle fibres in bees, leading to an impairment of flying activity.

Previous studies based on olfactory learning in the honeybee have shown the negative effects of fipronil on memory. Using conditioning of the proboscis extension reflex in restrained individuals, Decourtye et al. (2005) reported a decrease of the response level during the tests compared to the control group after chronic ingestion of fipronil (4.5 µg l^{-1} corresponding to a dose of 0.15 ng per bee per day).[14] El Hassani et al. (2005) showed that fipronil in acute topical application impaired olfactory learning of bees (0.5 ng per bee) and reduced their sucrose sensitivity (1 ng per bee).[13] The originality of our results consists in the demonstration of impact of fipronil on the orientation process which is a complex integrated function depending on phototaxis, learning of visual landmarks, memorization of the rule consisting in the association of the green mark to the right way. If our experiments would not allow conclusions about learning and memory impairment, they confirm the negative effects of the insecticide on the ability of bees to find a route.

While we cannot establish a direct link between previous results obtained in laboratory and the disorientation of foragers as suspected by beekeepers, our experimental data can tentatively be related to the field situation of bees exposed to fipronil.[13,14] In the field, foragers use landmark-based cues to navigate to a target as well as to return to the nest.[3] The learning flights that bees perform in order to memorise the location of a target typically cover a limited sector of space around the goal.[4] So, the memorized landmarks play a prominent role in path recognition during the next foraging trips. This work shows that the administration of 1 µg kg^{-1} of fipronil leads to disorientation of foragers. Unlike in the maze where the performances are based on the use of limited pertinent cues, the navigation in the field relies on several guidance mechanisms. Bees are capable of recognizing patterns in situations where local landmarks are not reliable.[28] Additional experiments are needed to establish whether foragers exposed to fipronil can negotiate a route in a complex environment or if they are lost, this being a possible cause in the drastic bee population losses as observed by beekeepers.

Acknowledgments

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Chlorantraniliprole (Rynaxypyr): A novel DuPont™ insecticide with low toxicity and low risk for honey bees (*Apis mellifera*) and bumble bees (*Bombus terrestris*) providing excellent tools for uses in integrated pest management

Axel Dinter[1]*, Kristin E. Brugger[2], Niels-Martin Frost[3], Michael D. Woodward[2]

[1]DuPont de Nemours Deutschland (GmbH), DuPont Str. 1, D-61352 Bad Homburg v.d.H., Germany.
[2]DuPont Crop Protection, Stine-Haskell Research Center, 1090 Elkton Road, Newark, DE 19714, USA
[3]DuPont Danmark ApS, Sköjtevej 26, DK-2770 Kastrup, Denmark
*Tel. +49(0)6175797211, Email: axel.dinter@dupont.com.

Abstract

Background: The effects on bees of chlorantraniliprole (DPX-E2Y45, DuPont™ Rynaxypyr), a new anthranilic diamide insecticide with a novel and very specific mode of action activating insect ryanodine receptors were investigated.

Results: Acute toxicity tests with chlorantraniliprole and the formulations, Coragen and Altacor, demonstrated low intrinsic toxicity to honey bees. Low risk for honey bees was demonstrated in semi-field tunnel tests with flowering *Phacelia* or wheat (with daily sprays of sugar solution to simulate honey dew) at application rates of Coragen of up to 60 g chlorantraniliprole/ha. Low potential of systemic exposure via pollen and nectar of honeybees to chlorantraniliprole was documented in a residue *Phacelia* tunnel trial with chlorantraniliprole applied to and mixed into bare soil. The impact of Altacor on bumble bees was studied in a greenhouse test in tomato at 40 g chlorantraniliprole/ha. Bumble bees directly over-sprayed during foraging activity with chlorantraniliprole or exposed to treated plants behaved as controls.

Conclusion: Chlorantraniliprole formulations provide excellent tools for integrated pest management (IPM) programmes to conserve pollinating honey bees and bumble bees.

Keywords: Chlorantraniliprole, Rynaxypyr®, insecticide, side-effects, honey bee, bumble bee, integrated pest management (IPM)

Introduction

Chlorantraniliprole (DuPont™ Rynaxypyr®) is a new anthranilic diamide insecticide developed worldwide by E.I. du Pont de Nemours and Company, Inc. with a novel and very specific mode of action. Chlorantraniliprole activates ryanodine receptors via stimulation of the release of calcium stores from the sarcoplasmic reticulum of muscle cells (i.e. for chewing insect pests) causing impaired regulation, paralysis and ultimately death of sensitive species[1]. The differential selectivity chlorantraniliprole has towards insect ryanodine receptors explains the outstanding profile of low mammalian toxicity[2]. Chlorantraniliprole is active on chewing pest insects primarily by ingestion and secondarily by contact. In Europe, Coragen® and Altacor® have been developed for foliar applications in top fruit, vegetable crops, grapes and potatoes at rates of 10 to 60 g chlorantraniliprole/ha, which are highly effective on many important pest insects[3]. The chlorantraniliprole formulations, Coragen and Altacor, were demonstrated to have negligible effects on numerous beneficial non-target arthropod species (e.g. the predatory mite *Typhlodromus pyri* or the parasitic wasp *Aphidius rhopalosiphi*) or to have rather low and transient impact on some slightly sensitive beneficial species[4]. This paper summarizes the current knowledge on effects of chlorantraniliprole and the formulated products, Coragen and Altacor, on honey bees and bumble bees.

Experimental methods

Effects of the active substance, chlorantraniliprole (also known as DPX-E2Y45 or Rynaxypyr), and two formulations, Coragen (200 g Rynaxypyr/L; DPX-E2Y45 20SC) and Altacor (350 g Rynaxypyr/kg; DPX-E2Y45 35WG), were studied using adopted test guidelines for honey bees (e.g. OECD or EPPO or CEB test methods) or with some modifications to address specific questions.

Acute honey bee testing

The intrinsic toxicity of the active substance chlorantraniliprole to the honeybee (Apis mellifera L.) (Hymenoptera, Apidae) was investigated in an acute oral and contact test following OECD Guideline No. 213 and No. 214[5,6]. Chlorantraniliprole is characterised by low solubility in water with a maximum solubility of 1 mg chlorantraniliprole/L water at 20°C. In the contact test, a stock solution of chlorantraniliprole was prepared in water at 1 mg active substance/L and either one 2-µL-droplet or one 5-µL-droplet were applied on the dorsal thorax of each honey bee to achieve maximal nominal doses of 2 and 5 ng chlorantraniliprole/bee. In the oral test – using the same water dilution approach – the bees were exposed to a dose of 27.4 ng chlorantraniliprole/bee. In another test acetone as an organic solvent was used to allow oral and contact testing at higher doses knowing that acetone is not used as an inert in any DuPont™ Rynaxypyr formulations. Oral and contact tests with the formulated products were performed without the use of any additional organic solvents.

Semi-field tunnel honey bee testing to assess effects from spray application

Coragen was chosen as the test substance for assessment of potential effects of chlorantraniliprole formulations under worst-case semi-field conditions because some sub-lethal effects were observed in the acute tests for this formulation, while no behavioural effects were observed for the Altacor formulation. Semi-field tests with small honey bee colonies that contained all brood stages at test start assessed the following effects during the pre- and post-application period: A) Mortality (counts of the numbers of dead honey bees in front of the hive and on sheets on the soil surface within the tunnel tests pre- and post-treatment), B) Foraging activity (visual counts of the numbers of foraging honey bees/m²), C.) Behavioural effects (visual assessments of the behaviour of the foraging honey bees on the crop and of honey bees around the hive), D.) Brood effects (assessments of the status of the honey bee colony regarding visibility of the honey bee queen and availability of eggs, larvae, pupae and adult honey bees inside the hive). Protocols fulfilled test guideline criteria although some observations were made at greater frequency than specified in guidelines in order to characterize potential changes as closely as possible.

Semi-field tunnel honey bee tests according to eppo 170-3: Three semi-field tunnel tests were conducted following the EPPO 170-3 test design with flowering *Phacelia tanacetifolia* Benth. as a model crop[7]. One trial each was performed in Germany and Spain with the formulated product Coragen and an application rate of 52.5 g chlorantraniliprole/ha. A third trial was conducted in France with the formulated product Coragen and an application rate of 60 g chlorantraniliprole/ha. The spray applications were all performed with hand-held boom sprayers at 400 L spray volume/ha during full flowering of the *Phacelia* crop and during foraging activity of the honey bees. Each trial had nine tunnels, three separated tunnels each for the control, chlorantraniliprole and toxic standard (260 g dimethoate/ha) treatments. Tunnels comprised an area of 50 to 60 m²/tunnel.

Semi-field tunnel honey bee tests according to ceb 230: Six semi-field tunnel tests following the CEB 230 test design were performed with flowering *Phacelia tanacetifolia* and winter winter wheat as model crops[8]. Three separate trials for each crop were performed in France with the formulated product Coragen and an application rate of 60 g chlorantraniliprole/ha. The spray applications were all conducted with hand-held boom sprayers and 200 or 300 L spray volume/ha during full flowering of the *Phacelia* crop or after wheat was sprayed with sugar solution to simulate honey dew. The control, one chlorantraniliprole and the toxic reference treatment (260 or 400 g dimethoate/ha) were sprayed while the honey bees were foraging, while another chlorantraniliprole tunnel was sprayed either in the late evening after daily honey bee flight or early in the morning before daily honey bee flight. There was one tunnel for each for the four treatments in line with the test guideline comprising an area of 64 to 80 m²/tunnel.

Semi-field tunnel honey bee test to quantify residue in bee matrices via systemic uptake from the soil

A semi-field study was conducted according to EPPO 170-3 and focused on residue analyses to determine whether chlorantraniliprole residues carried over in soil after applications at planting to the nectar or pollen in future flowering crops and to compare to the residues of an application made when bees were foraging[7].

Phacelia tanacetifolia was used a model crop because it is highly attractive to honey bees and is a fast-growing plant species with intensive roots growing in the top soil layer that was dosed with chlorantraniliprole. Residues of chlorantraniliprole were quantified in pollen and stomach nectar from foraging bees that returned to the hive, as well as residues in pollen, nectar and wax inside the hive after at-plant soil applications (to simulate soil residues from carry over) or foliar application. Soil was dosed at a rate that would simulate a long-term plateau concentration resulting from continuous maximum use over multiple years.

Treatments – with two separate tunnels each comprising each a crop area of about 100 m² – consisted of (a) a tap water control (C), (b) Coragen applied at 253.6 g chlorantraniliprole/ha and incorporated into the soil (10 cm depth) on the day of sowing of *P. tanacetifolia* followed by a second application at 60 g chlorantraniliprole/ha to the soil surface after sowing (equivalent to the estimated maximum soil exposure at the time of the study conduct) (T1) and (c) Coragen applied once at 60 g chlorantraniliprole/ha onto flowering *P. tanacetifolia* while honey bees were foraging (equivalent to worst-case exposure during foraging activity) (T2). Analytical dose verification in the soil demonstrated correct soil incorporation with chlorantraniliprole.

Forager bees were collected on 4 sampling days during exposure to flowering *P. tanacetifolia*. The samplings in all 3 treatments (C, T1 and T2) were conducted once before application of T2 and control (DAA-1; DAA = Days after application)) and three times after application of T2 and control: DAA+1, DAA+4 and DAA+7 (days after application). The bees were frozen (≤-18 °C) until the preparation of the honey stomachs and pollen loads from the forager bees and residue analysis. The pollen, nectar and wax samples (from the combs) were collected once before (DAA-1) and two times after application of T2 and control (DAA+1 and DAA+7). Each comb sample was taken from 3 spots per hive. For the sampling, pieces of combs with pollen and nectar were cut out from the combs by using a clean knife for each sample. During the assessment days it was tried to assure that the pollen and nectar collected was fresh collected from the *P. tanacetifolia* plot. The comb pieces for collecting pollen, nectar and wax were stored deep-frozen within 6 h after sampling (≤-18 °C) until residue analysis.

Bumble bee greenhouse testing

The objective of the study was to determine the effects of the insecticide Altacor on the bumble bee *Bombus terrestris* L. (Hymenoptera, Apidae) under semi-field conditions (greenhouse) in tomato based on general SETAC/ESCORT and EPPO 170-3 recommendations[7,9].

Young normal queen-right colonies each with 25 worker bumble bees were used. The colonies were matched for similar amounts of brood (larvae and pupae) at various stages of development. The colonies for the test were set-up in the greenhouse on 26 October 2007 in the late afternoon. The application of Altacor was performed in the greenhouse with flowering tomato plants. No bumble bee hives were used by the farmer before the introduction of the test hives. Four Altacor treatment and a control group were investigated: T1 = Altacor applied during foraging activity of the bumble bees, T2, T3 and T4 = Altacor applied 24 h, 48 h and 72 h before opening the hives, respectively; T2, T3 and T4 were applied with closed bumble bee hives and no bumble bees in the plots. On the day of the application in T1 and the control the bumblebees of all treatments were exposed (= start of exposure). After start of exposure the colonies were kept for 21 days in the greenhouse and assessed for mortality, foraging activity, condition of colonies and development of bumble bee brood. In each of the test item treatments the application of Altacor was performed at a rate of 114.3 g Altacor/ha (equivalent to 40.0 g chlorantraniliprole/ha) and at a target application volume of 1000 L/ha. Each treatment group comprised 4 greenhouse plots of at least 420 m^2 with one bumble bee hive/plot. The plots were separated by a net with a maximum mesh size of 5 mm. The study was located in Mazarron, region Murcia, Spain. The influence of Altacor was evaluated by comparing the results in the four Altacor treatments to the control regarding the following observations: Number of living and dead worker bees and larvae, foraging activity as measured by flower visits (bite marks), consumption of sugar solution, development of the bumble bee brood and condition of the colonies. The tomato blossoms were classified in 4 categories and each category received points (category 1: no bite mark = 1 point; category 2: 1-3 bite

marks/blossom = 2 points; category 3: > 3 bite marks/blossom = 3 points; category 4: blossom with brown pistil = 4 points).

Results

Acute honey bee toxicity

Low intrinsic honey bee toxicity of the active substance chlorantraniliprole and both demonstrated products, Altacor and Coragen, was demonstrated in acute oral and contact tests (Table 1). When the active substance chlorantraniliprole was tested up to the maximum solubility in water no significantly increased mortality or any sub-lethal effects of the honey bees were observed compared to the controls. The oral and contact LD_{50} values using water as solvent were >0.027 and >0.005 µg chlorantraniliprole/bee, respectively. Using acetone as organic solvent, which is not an actual inert in any of the DuPont chlorantraniliprole formulations, the oral and contact LD_{50} values were >104 and >4 µg chlorantraniliprole/bee, respectively. Under these artificial test conditions the honey bees were lethargic or apathetic following dosing but recovered during the following 48 to 72 h. Formulation testing did not require the use of any additional solvents. For Altacor the oral and contact LD_{50} value were >119.2 and 100 µg chlorantraniliprole/bee, respectively and no sub-lethal effects were observed at any dose tested. For Coragen the oral and contact LD_{50} value were >117.8 and 81.5 µg chlorantraniliprole/bee, respectively. Some honey bees treated with Coragen showed sub-lethal effects at the highest dosages tested. The oral and contact NOEC determined for Coragen were 63 and 12.5 µg chlorantraniliprole/bee, respectively. The hazard quotients (HQ) for both formulated product assuming the worst-case EU label rate of 60 g chlorantraniliprole/ha for any chlorantraniliprole formulation were all less than much less than one.

Table 1 Acute oral and contact toxicity of chlorantraniliprole and formulated products on honey bees and hazard quotients (HQ) [For the calculations of the HQs – defined as the maximum single application rate in g/ha divided by the LD50 in µg a.s./bee – the worst-case EU label rate of any chlorantraniliprole formulation of 60 g chlorantraniliprole/ha was considered].

Test material	**Oral LD_{50} (µg chlorantraniliprole per honey bee)**	**Contact LD_{50} (µg chlorantraniliprole per honey bee)**	**HQ_{oral}**	**$HQ_{contact}$**
Chlorantraniliprole (in water)	>0.027	>0.005	<2190	<12000
Chlorantraniliprole (in acetone)	>104	>4	<0.6	<15
Altacor	>119.2	>100	0.5	0.7
Coragen	>117.8	>81.5	<0.5	0.6

Results of semi-field tunnel honey bee tests to assess effects of spray application

Results of semi-field tunnel honey bee tests according to EPPO 170-3: Three fully replicated honey bee tunnel tests were conducted with spray application of Coragen at either 52.5 g (trials in Germany and Spain in 2004) or 60 g chlorantraniliprole/ha (trial in France in 2006). Results are summarized in Table 2. As an example, only the results of the trial with the highest application rate will be described in detail. On the day of application just before spray application high foraging activity was visually assessed with about 17 honey bees/m² in all three treatment groups. The foraging activity in the control and the Coragen treatment continued to be high following the spray application and was found to be >20 honey bees/m² the following day. Over the whole 7-day post-treatment assessment period no remarkable differences between the numbers of foraging honey bee/m² in the control and Coragen treatment was found, while in the toxic reference the numbers were low or zero (Figure 1).

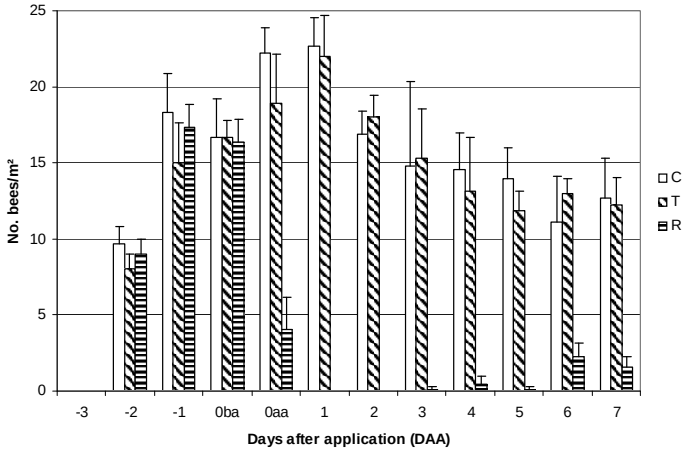

Figure 1 Mean honey bee flight intensity (number of honey bee/m² ± SD) in the control (C), Coragen at 60 g chlorantraniliprole/ha (T) and toxic reference treatment (dimethoate) prior to and after spray application during honey bee foraging activity in flowering *P. tanacetifolia* in France, 2006. (0ba = evaluation on the day of treatment shortly before application; 0aa = evaluation on the day of treatment after application)

During the pre-application period comparable numbers of dead honey bees were determined in all 3 treatment groups. On the day of treatment before application a mean number of 19.7 dead honey bees/tunnel was observed in the Coragen group compared to 22.0 dead honey bees/tunnel in the control and in the reference item treatment group, respectively. On the same day after application the mean number of dead bees in the Coragen treatment group was 24.7 dead honey bees/tunnel. In the control treatment a mean number of 12.7 dead honey bees/tunnel was found, while in the reference treatment group the mean number of dead honey bees increased to 605.0 dead honey bees/tunnel (Figure 2).

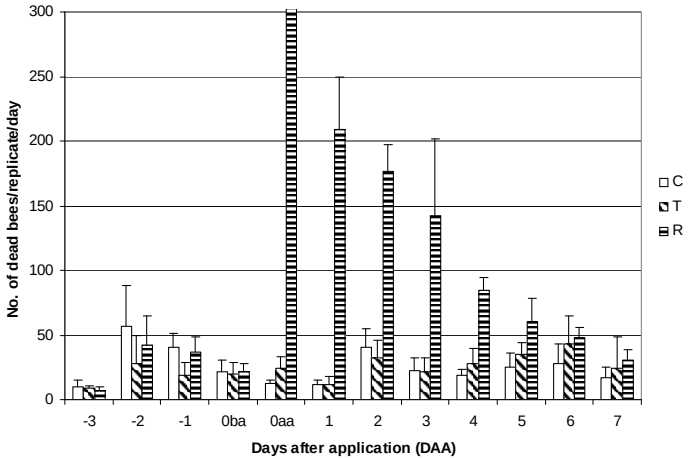

Figure 2 Mean number of dead honey bees/replicate tunnel/day (± SD) in the control (C), Coragen at 60 g chlorantraniliprole/ha (T) and toxic reference treatment (dimethoate) prior to and after spray application during honey bee foraging activity in flowering *P. tanacetifolia* in France, 2006. (0ba = evaluation on the day of treatment shortly before application; 0aa = evaluation on the day of treatment after application)

The mean post-application mortality was determined to be 27.7 dead honey bees/tunnel/day in the Coragen treatment group compared to 22.1 dead honey bees/tunnel/day in the control group and 169.5 dead honey bees/tunnel/day in the reference item treatment group. No significant differences were determined between pre-application mortality values of the Coragen, reference and control treatment (t-Test or Mann-Whitney test, $p > 0.05$). Furthermore no significant differences were found between pre- and post-application mortality data of the Coragen treatment group and the control group (t-Test, $p > 0.05$). The post-application mortality of the reference group was significantly different compared to the control group as well as of the Coragen treatment group (t-Test, $p < 0.05$). At the brood assessments carried out once before exposure (DAA-4) and 4-times after treatment (DAA+7, DAA+14, DAA+22 and DAA+28) all brood stages (egg stage, larval and pupal stage) in the colonies of all treatment groups were available. There were no differences between assessments of the strength of the colonies (number of bee ways between combs filled with honey bees) in the Coragen treatment group and control. The colonies of the control and Coragen treatment groups showed neither in the pre- nor in the post-application period noteworthy abnormal behaviour. In the toxic reference group abnormal honey bee behavior (cramping; collecting at the entrance) was noticed on the day of application after the spray application.

The results of all three EPPO honey bee tunnel trials with *P. tanacetifolia* are summarized in Table 2. Generally, honey bees resulted in mortality at levels comparable to the control group following exposure to Coragen spray solutions by direct overspray onto foraging honey bees. Also, there were no obvious differences found between the control and the Coragen treatment group regarding flight intensity, behaviour, colony strength or presence of queen, eggs, larvae or pupae.

Table 2 Results of three semi-field honey bee tunnel test according to EPPO 170-3 with Coragen sprayed during honey bee foraging activity in flowering *P. tanacetifilia*. (DAA = Day after spray application)

Country Year Rate	**Mortality Flight intensity Behaviour**	**Colony health (Hive assessment regarding colony strength and presence of queen, eggs, larvae and pupae)**
Germany 2004 52.5 g a.s./ha	No significant increase in mortality and no inhibition of flight intensity and no changes in individual behaviour compared to control	Colony strength not affected versus control All brood stages present on DAA+8
Spain 2004 52.5 g a.s./ha		Colony strength not affected versus control All brood stages present on DAA+22
France 2006 60 g a.s./ha		Colony strength not affected versus control All brood stages present on DAA+7, +14, +22, +28

Results of semi-field tunnel honey bee tests according to CEB 230: As an example for the three CEB trials conducted with wheat and daily sprays with sugar solution and simulate honey dew the results of a study conducted in 2006 in western France will be described. The daily mean flight intensity during the pre-application period varied between 4 to 16 honey bees/m² in the 4 different tents. On the day of treatment before spray application the honey bee flight activity with 11 to 16 honey bees/m² in the different treatments was far above the required level of > 3 honey bees/m² in all treatments. For all assessments the numbers of forager bees in both Coragen treatments were very similar to the control group. On the contrary in the reference tunnel the foraging activity decreased to almost nil during the whole post-treatment period (Figure 3).

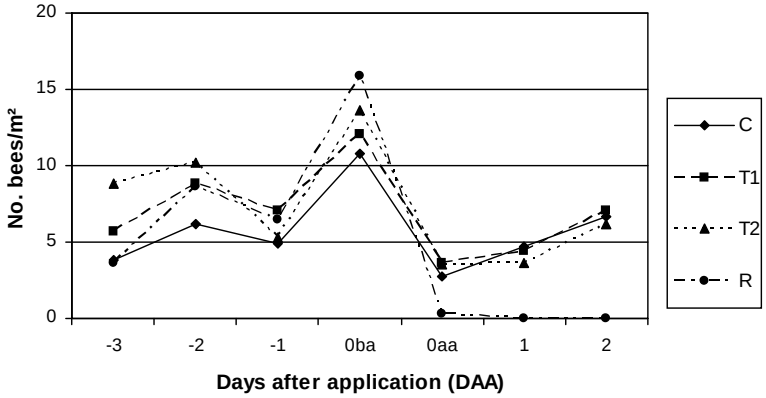

Figure 3 Honey bee flight intensity (number of honey bee/m²) in the control (C), Coragen at 60 g chlorantraniliprole/ha applied during honey bee flight (T1) or after daily bee flight (T2) and toxic reference treatment (dimethoate) (R) prior to and after spray application in winter wheat (sprayed daily with sugar solution to simulate honey dew) in France, 2006. (0ba = evaluation on the day of treatment shortly before application; 0aa = evaluation on the day of treatment after application)

The daily numbers of dead honey bee were homogenous and decreased from Day-3 to Day0 before application. The application of the toxic reference (400 g dimethoate/ha) induced a high peak mortality the day after spray application proving the sensitivity of the test system, while the numbers of dead forager bees in both Coragen treatments and in the control stayed all at about the same level as in the pre-treatment period (Figure 4). The two colony assessments before and after application did not show any significant changes due to Coragen applied during or after bee flight relative to the control. No symptoms of poisoning or abnormal behaviour were recorded during the whole trial period in any of the treatment groups, i.e. Coragen treatment groups relative to the control.

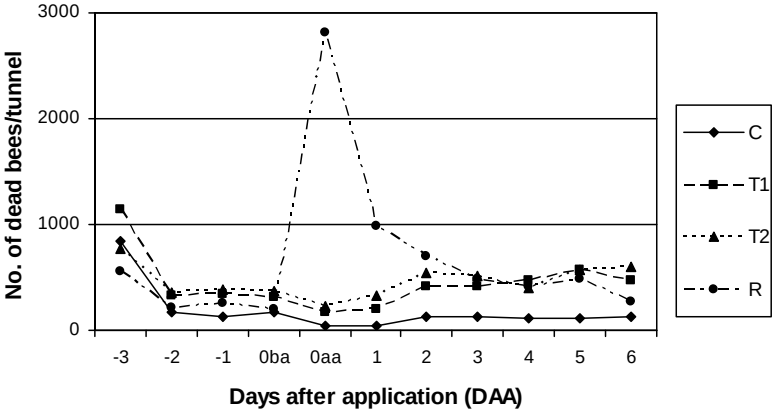

Figure 4 Number of dead honey bees per tunnel and day in the control (C), Coragen at 60 g chlorantraniliprole/ha applied during honey bee flight (T1) or after daily bee flight (T2) and toxic reference treatment (dimethoate) (R) prior to and after spray application in winter wheat (sprayed daily with sugar solution to simulate honey dew) in France, 2006. (0ba = evaluation on the day of treatment shortly before application; 0aa = evaluation on the day of treatment after application)

In all six semi-field honey bee tunnel trials following the CEB 230 design – either conducted with flowering *Phacelia* or wheat as crop – comparable levels of honey bee mortality were determined for the Coragen treatments (sprayed during honey bee foraging activity or outside of flight activity) and the control group. Also, there were no obvious differences found between the control group and the two Coragen treatment groups regarding flight intensity, behaviour, colony strength or presence of queen, eggs, larvae or pupae.

Results of semi-field tunnel honey bee test to quantify residue in bee matrices via systemic uptake from the soil

Chlorantraniliprole residue concentrations were determined in nectar and pollen of *P. tanacetifolia*, which was grown in soil treated with chlorantraniliprole, and in bee wax produced by honey bees foraging on the exposed plants. Residue concentrations in pollen, nectar and wax were determined following an application with chlorantraniliprole onto honey bees foraging on flowering *P. tanacetifolia* plants. Results are summarized in Table 3. Chlorantraniliprole residue concentrations in pollen and nectar foraged from plants grown in chlorantraniliprole pre-treated soil were found only in samples taken from bee legs and the honey stomach of forager bees collected in front of the hive and were significantly lower than residues resulting from direct spray application at 60 g chlorantraniliprole/ha. No chlorantraniliprole residues have been determined inside the hives of honey bees foraging on plants growing on chlorantraniliprole pre-treated soil, indicating that honey bees are not markedly exposed to systemic residues of chlorantraniliprole in plants.

Table 3 Maximum chlorantraniliprole residues (mg/kg) determined in nectar (from bee stomach content) and pollen (from honey bee legs) sampled from forager honey bees (outside hive) and determined in nectar, pollen and wax from honey bee combs inside honey bee hives from colonies kept in *Phacelia* control tunnels (n = 2) and, *Phacelia* tunnels following soil application at sowing with chlorantraniliprole at 253.6 g chlorantraniliprole/ha and following spray application at 60 g chlorantraniliprole/ha during honey bee foraging activity in flowering *Phacelia*.

		Forager bees - outside hive		Comb samples - inside hive		
Treatment	**DAA**	**Nectar**	**Pollen**	**Nectar**	**Pollen**	**Wax**
				(mg chlorantraniliprole/kg)		
Control	-1	**	**	**	*/**	**
	+1	**	**	**	*/**	**
	+3	**	**	ns	ns	ns
	+7	**	**	**	**	*/**
Soil application	-1	*	*	**	**	**
	+1	*	*	**	**	**
	+3	0.0032	0.0010	ns	ns	ns
	+7	*	0.0018	**	**	**
Spray onto foraging bees in flowering *Phacelia*	-1	**	**	**	**	**
	+1	0.0330	2.6010	0.0472	2.8630	0.0105
	+3	0.0096	0.7633	ns	ns	ns
	+7	0.0036	0.2643	0.0013	0.1080	0.0757

*: < LOQ = Limit of Quantification = 0.001 mg chlorantraniliprole/kg; **: < LOD = Limit of Detection = 0.0003 mg chlorantraniliprole/kg, ns: not sampled

Results on bumble testing under commercial greenhouse conditions

The mean numbers of dead larvae, pupae and adult bumble bees per day and hive in the 4 subplots before application were 1.6 in the control treatment and 1.6, 1.4, 2.8 and 1.3 in the Altacor treatments T1, T2, T3 and T4, respectively. From DAA0 until DAA+7 after the application in C and T1 the mean number of dead larvae, pupae and adult bumble bees per day and hive of the four replicates, respectively, was between 0.6 and 1.0 in all treatment groups without any differences between the treated and the control plots (Figure 5).

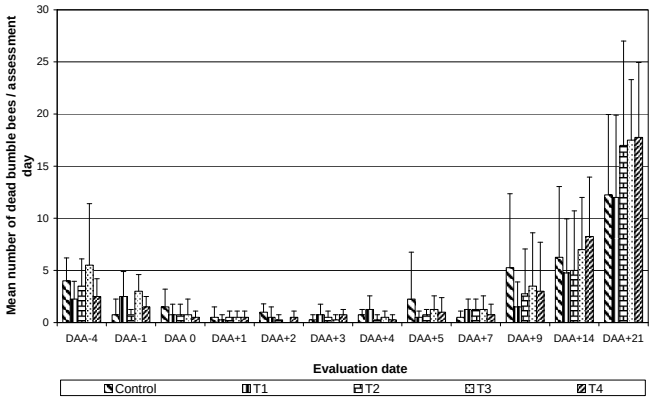

Figure 5 Mean number of dead bumble bees per day (adult workers and larvae) (± SD) collected in the field, in front and inside the four colonies of the control and the Altacor treatments T1 to T4. (T1 = Altacor applied during foraging activity of the bumble bees, T2, T3 and T4 = Altacor applied 24 h, 48 h and 72 h before opening the hives, respectively; T2, T3 and T4 were applied with closed bumble bee hives and no bumble bees in the plots.)

From DAA+9 mortality increased in all treatment groups in parallel due to the increasing strength of the colonies and the decreasing food availability in the greenhouse, and was mainly caused by larval death. During the entire assessment period after application the mean number of dead bumble bees per day and hive in the 4 subplots was 3.1 in the control treatment, 2.4, 2.9, 3.3 and 3.3 in the Altacor treatments T1, T2, T3 and T4, respectively. No statistically significant differences between the control and any of the Altacor treatment groups were calculated (Dunnett's t-test and Bonferroni U (Holms) Exact test, two-sided; $p \leq 0.05$). In all treatment groups the bumble bees started immediately (on the day after set-up in the greenhouse) pollinating the crop and leaving on flowers visited so called "bite marks" and a continuous increase of the pollination activity (increase of the number of points) was observed during the course of the study from DAA-4 up to DAA+2 when pollinating activity approached a maximum (Figure 6).

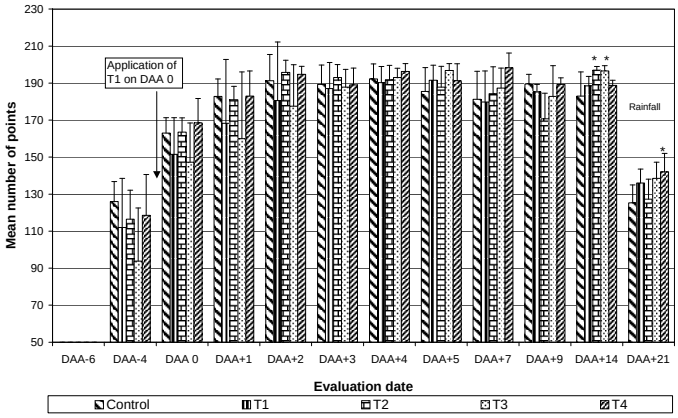

Figure 6 Foraging activity of bumble bees given as mean number of points[#] in the control and the Altacor treatments T1 to T4. (T1 = Altacor applied during foraging activity of the bumble bees, T2, T3 and T4 = Altacor applied 24 h, 48 h and 72 h before opening the hives, respectively; T2, T3 and T4 were applied with closed bumble bee hives and no bumble bees in the plots.). (* = significantly higher than the control (Dunnetts t-Test ($p \leq 0.05$, two-sided)) ([#] = Category 1: no bite mark = 1 point; Category 2: 1-3 bite marks/flower = 2 points; Category 3: > 3 bite marks/Flower = 3 points; category 4: brown Pistil = 4 points.)

From DAA+2 the pollinating activity remained at high level until end of the exposure period on DAA+21. Foraging activity was statistically significantly higher than in the control group on DAA+14 in the Altacor treatment T2 and T3, and on DAA+21 in T4 (Dunnett's t-test, two-sided; $p \leq 0.05$). On DAA+21 foraging activity was relative low, but comparable in all treatment groups due to bad weather conditions.

The mean sugar solution uptake of the bumble bees was similar in the control treatment and in the Altacor treatments T1, T2, and slightly higher in T3 and T4 (Figure 7). The mean sugar solution consumption of the bumble bees from the set-up of the colonies until the last day of exposure was 580 g in the control and 584 g, 593 g, 670 g and 708 g in the Altacor treatment T1, T2, T3 and T4, respectively. No statistically significant differences between the control and any of the Altacor treatment groups were calculated (Dunnett's t-test, Bonferroni U (Holms) Exact test, Welch Bonferroni Holms corrected, two-sided, $p \leq 0.05$).

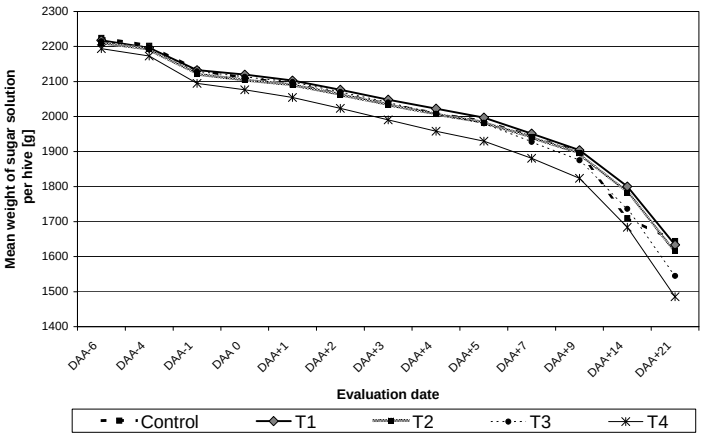

Figure 7 Mean weight of the sugar solution bags of the four colonies of the control and the Altacor treatments T1 to T4. (T1 = Altacor applied during foraging activity of the bumble bees, T2, T3 and T4 = Altacor applied 24 h, 48 h and 72 h before opening the hives, respectively; T2, T3 and T4 were applied with closed bumble bee hives and no bumble bees in the plots.)

During the assessment period from DAA-6 up to DAA0 slight fluctuations in the weight of the colonies were observed in all treatment groups. From DAA0 to the last assessment date on DAA+21 the mean weight in the colonies of the treatment groups increased clearly (Figure 8). In view of the total observation period from DAA-6 until DAA+21 the colonies of all treatment groups increased their mean weight, i.e. the colonies of the control treatment by 97 g and 109 g, 109 g, 127 g and 131 g in the Altacor treatments T1, T2, T3 and T4, respectively. No statistically significant differences between the control and any of the chlorantraniliprole treatment groups were calculated (Dunnett's t-test, Bonferroni U (Holms) Exact test, two-sided, $p \leq 0.05$).

When the final brood assessment was carried out 22 days after the start of exposure all hives were in the process of disposing their original living old queens which had been in the hives since the start of the study. Additionally, one colony of C, two colonies of T1, two colonies of T2, three colonies of T3 and three colonies of T4 had young newly hatched queens, respectively. Two colonies of the control, one colony of T2 and one colony of T4 had unhatched queen pupae. In one colony of the control and one of T3 no eggs were found. In all other colonies the presence of the queen, eggs, larval stages and pupae showed that the colonies were in good condition. The mean number of worker bees in the colonies of the treatment groups at the final brood assessment was 100 in the control treatment and 119, 129, 159, and 153 in the Altacor treatments T1, T2, T3 and T4, respectively. No abnormal differences in brood development, which could be attributed to the influence of chlorantraniliprole were observed between the control and the Altacor treatments.

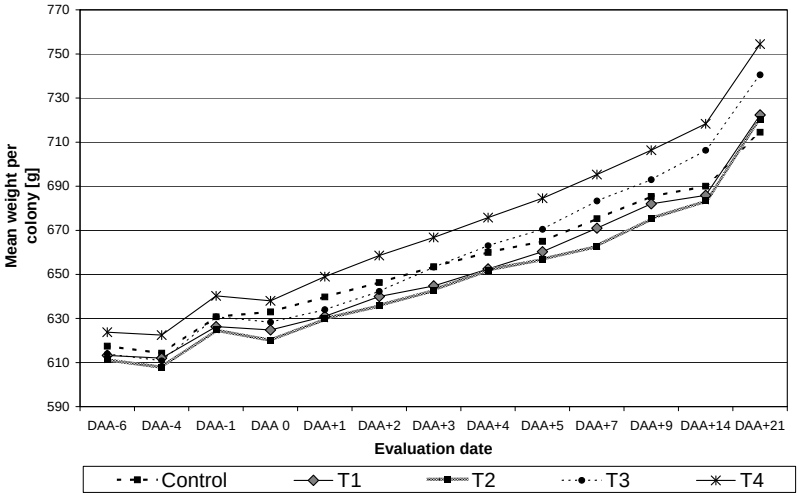

Figure 8 Mean weight of the four bumble bee colonies of the control and the Altacor treatments T1 to T4. (T1 = Altacor applied during foraging activity of the bumble bees, T2, T3 and T4 = Altacor applied 24 h, 48 h and 72 h before opening the hives, respectively; T2, T3 and T4 were applied with closed bumble bee hives and no bumble bees in the plots.)

Discussion

Chlorantraniliprole exhibited low intrinsic toxicity for honey bees. This is in line with findings of low sensitivity for other hymenopteran species, e.g. the parasitic wasp, *Aphidius rhopalosiphi*, for which the LR50 and ER50 values – based on mortality and reproduction – were both > 750 g chlorantraniliprole/ha on inert substrate (glass plates) for Coragen and Altacor[4]. When the active substance chlorantraniliprole was tested up the maximum water solubility of 1 mg chlorantraniliprole/L survival and behaviour of treated honey bees were unaffected. The same observations and lack of any lethal or sub-lethal effects were made for the formulated product Altacor, the wet-able granule, up to maximum tested rates (oral and contact) of >119.2 and 100 µg chlorantraniliprole/bee, respectively. When chlorantraniliprole was applied in combination with the organic solvent, acetone, sub-lethal effects were observed. Also for the liquid formulation, Coragen, some honey bees showed sub-lethal effects, but only at higher dosages tested. From these observations it can be concluded that chlorantraniliprole is unlikely to reach and affect the target sites, ryanodine receptors, in honey bees, when dissolved in watery solutions. The calculated hazard quotient (HQ) values for both formulations Coragen and Altacor were all <1 and far below the EU-relevant trigger value of 50 predicting high margins of safety for honey bees in flowering crops. Also for application rates of chlorantraniliprole above the EU-intended uses the HQ values will remain below the EU HQ trigger of 50 indicating low risk for honey bees for the world-wide intended uses of chlorantraniliprole.

The prediction of low risk for honey bees due to the uses of chlorantraniliprole were confirmed in numerous semi-field tunnel tests with Coragen conducted under worst-case exposure conditions in various locations in Germany, France and Spain and in different years. In these tests it was found that the spray application of chlorantraniliprole performed during full foraging activity or exposure to spray deposits (treatment outside foraging) in flowering crops (*Phacelia*) or crops bee-attractive due to honey dew (simulated via daily sprays of sugar solutions) did not have any effects regarding all parameters assessed, i.e. mortality, foraging activity, behaviour or condition of the colonies and development of honey bee brood assessed for up to 28 days after treatment relative to water treated controls for rates up to 60 g chlorantraniliprole/ha. No negative

effects were found in an early *Phacelia* tunnel screening test sprayed at 75 g chlorantraniliprole/ha during foraging activity of honey bees (DuPont, unpublished).

Exposure to chlorantraniliprole residues from carry over in soil after applications at planting to the nectar or pollen in future flowering crops was much lower than residues from direct exposure of honey bees via direct spray application. No quantifiable amounts of chlorantraniliprole residues were found inside the hives via worst-case soil dosing and simulation of a long-term plateau concentration resulting from continuous maximum use over multiple years.

Chlorantraniliprole was compatible with bumble bees as crop pollinators in greenhouses. Altacor when applied during foraging activity or 24 h, 48 h or 72 h before opening of the hives of the bumble bee, *B. terrestris*, at a rate of 40 g chlorantraniliprole/ha (maximum recommended rate) and an application volume of 1000 L per ha did not have any effects regarding all parameters assessed, i.e. mortality, foraging activity, condition of colonies and development of bumble bee brood relative to the water treated control. The low toxicity of chlorantraniliprole was also confirmed for another bumble bee species, *Bombus impatiens* Cresson, an important indigenous pollinator in North America[10]. Adult *B. impatiens* worker bees didn't shown any increased mortality (0% mortality corrected for control) when exposed via direct spray contact (Potter tower) to spray solutions (19:1 acetone: olive oil solution) containing 0.001, 0.01 and 0.1 % chlorantraniliprole, while other insecticides significantly increased mortality to levels >80% mortality tested at the same three concentrations (imidacloprid) or at the 2 hightest tested concentrations of 0.01 and 0.1 % (metaflumizone and abamectin). Chlorantraniliprole at up to 900 ppm did not affect survival, infectivity, and reproduction of the entompathogenic nematode, *Heterorhabditis bateriphora*, offering e.g. a highly effective option for remedial white grub control in greenhouses[11].

Conclusions

Chlorantraniliprole (DuPont™ Rynaxypyr) and its formulated products, Coragen and Altacor demonstrated low intrinsic toxicity for honey bees and bumblebees. In worst-case tunnel and greenhouse trials no significant effects on pollinating bees were found, even when bees were directly over-sprayed during foraging activity. This indicates a high margin of safety for honey bees and bumble bees for the uses of chlorantraniliprole and its formulated products, Coragen and Altacor, in flowering crops and in succeeding crops. As chlorantraniliprole has proven to have negligible effects on numerous beneficial non-target arthropod species or to have a rather low and transient impact on some beneficial species, too, Coragen and Altacor provide excellent tools for integrated pest management (IPM) programmes. In line with Good Agricultural Practice and to avoid unnecessary contamination of pollinators spray applications should always be made when pollinators are not foraging or after daily bee flight.

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Honey bee brood ring-test: method for testing pesticide toxicity on honeybee brood in laboratory conditions

Pierrick Aupinel[1*], Dominique Fortini[1], Bruno Michaud[1], Piotr Medrzycki[2], Erica Padovani[2], Dunja Przygoda[3], Christian Maus[3], Jean-Daniel Charriere[4], Verena Kilchenmann[4], Ulrike Riessberger-Galle[5], Jutta Juliane Vollmann[5], Lukas Jeker[6], Martina Janke[7], Jean-François Odoux[1], Jean-Noël Tasei[1]

[1]Unité expérimentale d'entomologie, INRA, Le Magneraud, BP 52, 17700 Surgères, France
[2]Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Unità di Ricerca di Apicoltura e Bachicoltura, Via di Saliceto 80, 40128 Bologna, Italia
[3]Bayer CropScience AG, Ecotoxicology, 6620, Alfred-Nobel-Str. 50, 40789 Monheim, Germany
[4]Agroscope Liebefeld-Posieux ALP, Schwarzenburgstrasse 163, CH - 3003 Berne Switzerland
[5]Institut für Zoologie, Abteilung Stoffwechselphysiologie und Verhalten, Universitätsplatz 2, A-8010 Graz, Austria.
[6]Harlan Laboratories Ltd. Zegliwed 1 CH-4452 Itingen Switzerland
[7]LAVES Laves Institut für bienenkunde Celle, Germany
*Tel.: +33(0)546683002, fax: +33(0)546683062, e-mail: Pierrick.Aupinel@magneraud.inra.fr

Abstract

The Experimental unit of entomology (INRA, France) developed a new *in vitro* method to assess effects of pesticides on honey bee larvae. The method consists in rearing bee larvae in plastic cells. The larvae are fed with diet containing 50% of fresh royal jelly and 50% of an aqueous sugar and yeast extract solution, and reared in an incubator at 35 °C and 96% relative humidity. According to that method, 9 tests (7 in 2008 and 2 in 2005) were carried out in 7 laboratories and different countries. The objective of these trials was to assess the LD_{50} for dimethoate 48 hours after an acute exposure.

The LD_{50} values ranged from 1.5 µg a.i./larva to 8.8 µg a.i./larva, with 2 tests with particularly high values (5.0 and 8.8 µg a.i./larva). In 7 tests, these values ranged from 1.5 µg a.i./larva to 3.1 µg a.i./larva. Such variability may be due to the colony origin, the season and larva heterogeneity at grafting. Solutions are proposed to improve the method through the continuation of the ring test.

Keywords**:** *Apis mellifera*, brood, *in vitro* test, dimethoate

Introduction

According to the guidelines of the European Union (91/414 EEC) [1] a brood feeding test is requested in cases where honeybees (*Apis mellifera* L.) are exposed to treatments of insect growth regulators (IGR). The official recommended method is that of Oomen et al.[2] which is an in-hive method where experimental bees are free-flying colonies. The artificial contamination is ensured by a syrup feeder (1 litre) fitted to the hive for 24h. Due to environmental variations to which bee hives are subjected under open-field conditions, the method may in some cases not be capable of providing an accurate measurement of intrinsic toxicity. Moreover, certain details of the method have been challenged, e.g. there was concern that the tested product may be stored in the combs and not immediately dispensed to the brood by nurse bees. Then, if the hives are not set up in sufficient isolation, exposure to products may also be modified by dilution with nectar collected by foragers from attractive crops in the surroundings. In addition the method provides no quantitative data on individual larvae since no measurement of the product ingested by larvae is feasible. At last, this method has never been validated and therefore should be seen with caution. A new method was recently established by Schur et al.[3] and is meanwhile implemented as OECD Guidance Document 75[4] that consists in testing the effect of pesticides on honey bee brood in semi-field conditions. This test was validated through a ring test and could be considered as a second tier test on bee brood in the risk assessment scheme.

A new *in vitro* test which could be used as a tier 1 test in the risk assessment scheme was described by Aupinel et al.[5-7] This standardized test permits an accurate measurement of the quantity of the tested substance to which a larva is exposed and can be run in low cost conditions compared to a semi field or field test. For these reasons, it can be used as a preliminary screening test. This test was presented during the last ICPBR symposium "Hazard of pesticides to Bee" in York in 2005 where it was decided to run a ring test in order to validate it.

The objective of this work is to test and validate this *in vitro* laboratory method in order to complete the testing scheme with a tier 1 honey bee brood test.

Experimental methods

Testing conditions

The main rearing principles for honeybee larvae were described in Aupinel et al.[5] Three diet compositions (A, B, C) were used, all composed by aqueous sugar and yeast extract solution and fresh royal jelly (1+1 by weight). The composition of the aqueous part and the amount supplied to each larva, according to the rearing day, is described in Table 1.

Table 1 Quantity and composition of the diet provided to reared larvae.

Diet	Rearing day	Diet amount supplied to each larva (µL)	Composition of the aqueous part (mg g^{-1})		
			D-glucose	D-fructose	yeast extract
A	1	20	120	120	20
B	3	20	150	150	30
C	4, 5, 6	30, 40, 50 (respectively)	180	180	40

Eggs of the same age were obtained from healthy colonies where queens were confined on a comb in excluder cages for 30 hours. These cages permitted worker bees to move freely from the encaged comb to other parts of the colony in order to stimulate egg laying and feeding of the larvae. After removing the queen, the comb was left in its cage in the colony for three days. Then the frame was removed from the hive and brought into the laboratory for grafting the larvae with a fine paintbrush or another suitable grafting device into traditional plastic queen starters (Nicoplast©) that had been previously disinfected for 30 minutes in water solution of methyl benzonium chloride (MBC) (4 g L^{-1}). The diet A (20 µl) was deposited at the bottom of each cell before grafting. The cells were set in the wells of a 48-well cellular culture plate. A dental roll impregnated with glycerol (155 g L^{-1}) diluted in aqueous solution of MBC (4 g L^{-1}) was placed at

the bottom of the wells before introducing the cells. From day 1 to day 7 (or 8, according to diet consumption) the larvae plates were kept in an incubator at 35 °C and 96% RH, and were taken out once a day for feeding except at day 2. After day 7 (or 8) the larvae were moved to a second incubator at 35°C and 80% RH. Before emergence time the plates were placed in plastic boxes fitted with a suitable feeding device (e.g. a bird feeder) containing syrup.

Experimental design

The aim of the ring test was to assess the larval LD_{50} with dimethoate (technical) 48h after an acute exposure at the age of 4 days. We used dimethoate originated from the same lot, characterised by a purity of 99%. Dimethoate was chosen for three reasons:

- It produces acute effects compared with substances like fenoxycarb (toxic standard in the semifield brood test, OECD Guidance Document 75) that induces effects later in the developmental process.[6]
- We have good experience of its use in the presented test design
- It is the standard reference compound for acute toxicity test on adults.

In 2008 the ring test was carried out by seven laboratories from different countries that ran a total of 7 tests with a minimum of 3 valid trials each. Two validity criteria were set: control mortality lower than 15% at D6; and successful hatch of adults in at least the control group. The dimethoate dilutions where prepared in order to expose larvae to doses ranging from 0.83 µg/larva to 13.20 µg/larva with a spacing factor of 2. Each trial replicate run consisted in 6 plates of the same size with a minimum of 30 larvae per plate at D4, and all larvae preferably originating from the same hive. Thus, 5 treated plates and 1 control plate were used in each replicate.

Results obtained in 2005 in the same experimental conditions, except for one where the tested concentrations ranged from 0.40 µg/larva to 6.6 µg/larva, were added in order to increase the amount of data. No test was run between 2005 and 2008.

Observations and LD_{50} assessment

The number of dead larvae was recorded at D4 (before the sample size adjustment), at D6 (48h after start of exposure), and at D22 after adult emergence. An immobile larva or a larva which did not react to the contact of the paintbrush was recorded as dead. The LD_{50} and 95% confidence limits from individual trials were obtained by the standard method of linear regression of the logit transformation of percentage of mortality in $\log_{10}$ dose (µg a.i./larva), adjustments being made for control mortality using Abbott's correction.

Results

31 trials for 9 tests were run in 2005 and 2008 by 7 institutions in 5 countries (Table 2). More than one colony was generally used for the tests, except for the test D and E. In the test D, the three required trials were run within one week whereas it took more than one week between two trials in the other tests. Both Carnica and Ligustica bee subspecies were used according to the standard practice of the respective laboratory.

Table 2 Dates of the tests, bee races and number of hive used for the tests. A-G: tests run in 2008; H-I: tests run in 2005.

Test	Starting date of trials	Bee subspecies	Number of colonies providing larvae
A	Aug. 4, 25; Sept. 8	Carnica	2
B	May 23, 30; June 6	Carnica	5
C*	May 23, 30; June 20; Sept. 19	Ligustica	4
D	Apr. 28, 30; May 2	Carnica	1
E	July 21, 28; Aug. 11	Carnica	1
F	May 30; June 20, 27	Ligustica	3

Test	Starting date of trials	Bee subspecies	Number of colonies providing larvae
G	June 6, 20; July 4, 18; Aug. 8	Carnica	2
H*	May 27; June 3, 10	Ligustica	3
I*	June 24; Sept. 9, 16, 23	Ligustica	4

* tests run in the same institute

The results of all the trials respected the defined validity criteria (Figures 1-2). In all tests adults emerged in control samples. In only three trials the control mortality at D6 exceeded 10% and was never higher than 15%. In 25 trials the control mortality at D6 didn't exceed 5%. In 20 trials more than 50% emerged adults were observed in the control.

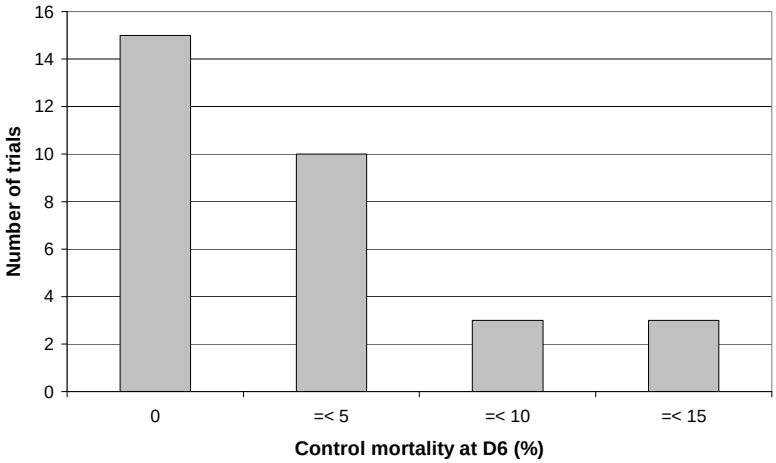

Figure 1 Number of trials characterised by different control mortality rates at D6

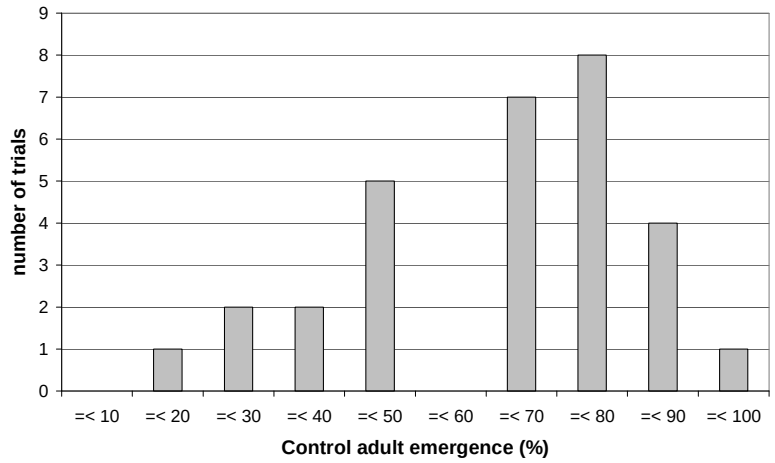

Figure 2 Number of trials characterised by different control adult emergence rate

The LD_{50} arithmetic mean values diverged among the different tests (Figure 3). In the tests C and D we noted particularly high mean LD_{50} values (5.0 and 8.8 µg a.i./larva respectively), whereas in the remaining 7 tests, the mean LD_{50} ranged from 1.5 µg a.i./larva (test E) to 3.1 µg a.i./larva (test F). We also noted a larger variability in individual LD_{50} in test D. No significant difference (Kruskall-Wallis test, H = 0,17, df = 1, P = 0,677) was noted between mean LD_{50} calculated for the Carnica and the Ligustica subspecies (2.51 µg a.i./larva, 2.99 µg a.i./larva respectively).

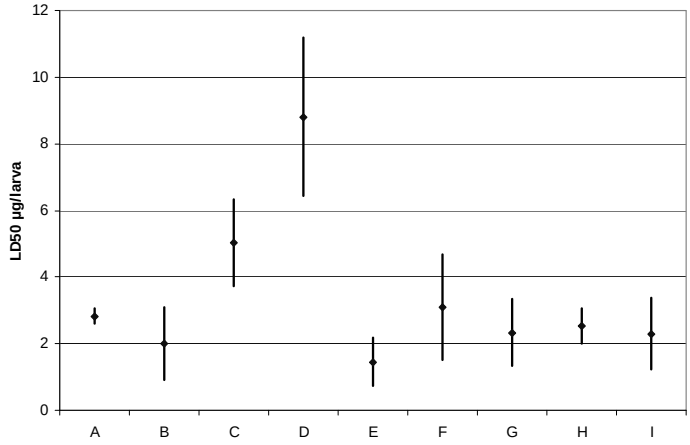

Figure 3 Mean LD50 for each test (± standard deviation)

There were no significant relationships between the LD_{50} values and the control mortality rate observed at D6 (Figure 4) and the control adult emergence at D22 (Figure 5) so that these variables cannot explain LD_{50} variation.

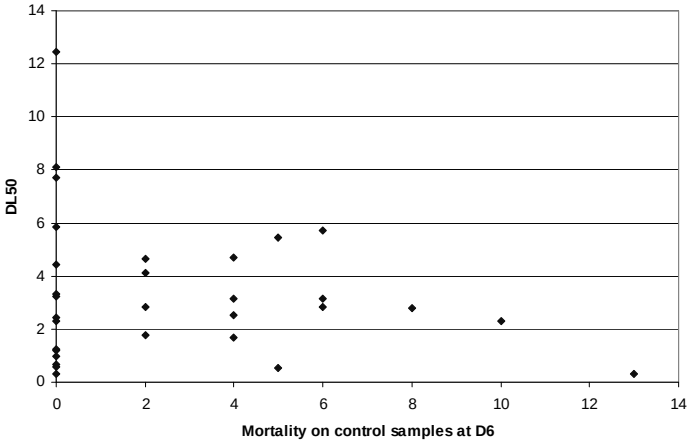

Figure 4 Relationship between LD_{50} and % mortality in the control samples at D6

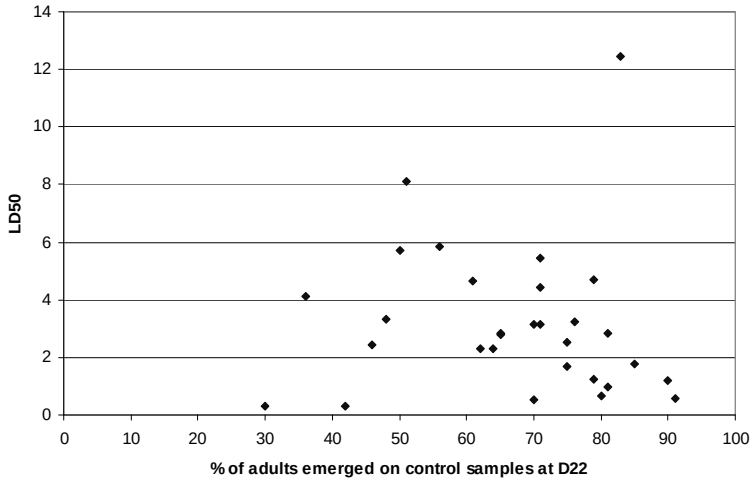

Figure 5 Relationship between LD_{50} and % adults emergence at D22 in the control samples.

Discussion and conclusions

These results firstly show that a large number of valid trials can be run by different laboratories and then demonstrate clearly that this method is accessible with some basic material. This is the first condition in order that a method could become a routine test. For 7 of the tests, the mean LD_{50} ranges with a factor of 2 that is lower to what was observed by Gough et al.[8] in oral tests with dimethoate at 48h on adult bees, run in the same laboratory who noted LD_{50} values that ranged from 0.100 to 0.318 µg a.i./bee. Moreover, the LD_{50} calculated in these tests were close to the values already published [6,7] (1.93 and 1.80 µg a.i/larva). In two tests (C and D), and in particular one of them (D), larvae revealed a high level of resistance to dimethoate. Many hypotheses can be suggested to explain such a phenomenon. It has to be noted that the test D was conducted under particular conditions in comparison to the other tests. The three trials were run within only one week with the larvae from the same colony. If we admit, as we already noted in precedent experiments, that tolerance to a pesticide may intrinsically vary according to the colony, we can challenge whether the three trials run in this test were true replicates. This effect may be reinforced by the fact that the test was run over a very short time, so that no eventual time effect could be eliminated. In order to avoid potential colony and time effects, it is recommended that different colonies are used for each test, and to run the three trials at intervals of a minimum of one week.

The last probable reason for such difference may be related to the egg-laying behaviour of the queen. The queen is encaged for 30 hours in order to obtain a large number of homogenous young larvae. According to the laying precocity of the queen, the difference of age between larvae originated from different colonies may reach 24 hours. Larvae issued from an early laying queen will be older than larvae issued from a late laying queen when they are exposed. Such a difference of age and instars may explain difference in sensitivity to an insecticide. This hypothesis will have to be verified. One way to avoid such effect would be to encage the queen for a shorter time in order to reduce the queen laying period. For this reason, it has been decided during the 2008 ICPBR meeting to improve the method and continue the ring test in 2009. In spite of this point that has to be specified, this test could then be carried out routinely as Tier 1, and complete the semi field test described by Schur *et al.* [3] in the risk assessment scheme. Moreover, it has to be noted that these two tests on honey bee brood are the only ones that are validated or in the process of being validated.

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Comparison of two methods to assess effects of insecticides on hypopharyngeal gland development of honey bee

Dominique Fortini, Bruno Michaud, Pierrick Aupinel

Unité expérimentale d'entomologie, INRA, Le Magneraud, BP 52, 17700 Surgères, France

Abstract

Hypopharyngeal glands (HPG) are the main organs responsible of royal jelly secretion. The size of the HPG is age[2, 4] and food protein[5, 7] dependent, and correlated to the amount of secretion[2], and the weight of the head[5]. Their development can be assessed with a microscope by measuring the *acini* diameter after dissection.. This very useful method[1, 3, 5, 6] has some inconveniences: it requires dexterity to extract the gland, and the diameter of the *acini* is difficult to measure because of its pear shape. In order to assess the HPG development, total protein of the gland can be measured with the Bradford method[7, 8, 9], but this also requires to extract it from the head.

The development of the HPG may be also affected by substances known for their insecticide effects like soybean tripsin inhibitor [8, 9].

The objective of this work is to compare two methods for assessing the effects of insecticides on HGP development. The first one consists in measuring the *acini* diameter, and the second one in measuring the total protein of the head. The measurements are made on bee nurses intoxicated during 10 days with sublethal doses of dimethoate.

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V. Honey bee poisoning incidents and monitoring schemes

Review of honeybee pesticide poisoning incidents in Europe – evaluation of the hazard quotient approach for risk assessment

Helen M Thompson[1*], David Thorbahn[2]

[1] CSL, Sand Hutton, York, UK YO41 1LZ
[2] Biologische Bundesanstalt für Land- und Forstwirtschaft, Untersuchungsstelle für Bienenvergiftungen, Messeweg 11-12 , 38104 Braunschweig, Germany
*email: h.thompson@csl.gov.uk; Tel: 44 1904 462515; fax 44 1904 462515

Abstract

Background: Honeybee risk assessment is required in Europe for all pesticides where bees may be exposed. This is well established for sprayed products where the hazard quotient (HQ), calculated by dividing the application rate of the sprayed product active ingredient by the LD50, is less than 50 the product is considered safe to bees (unless it is an IGR). In the UK, Germany and the Netherlands post-registration monitoring schemes on the poisoning of honeybee by pesticides collate data on honeybee incidents.

Results: The incident schemes have been invaluable in identifying agronomic practices resulting in honeybee mortality and changes have been made to labelling to address such issues. The decrease in the numbers of incidents reported supports the assertion that such schemes have positively contributed to the regulatory process and also provide confidence in the risk assessment approaches.

Conclusion: This review of incidents in Europe over the last 25 years suggests that the HQ approach to risk assessment for honeybees offers an appropriate level of protection.

Keywords: honeybee, pesticide, hazard quotient, risk assessment

Introduction

In Europe EU Directive 91/414 requires honeybee risk assessment to be undertaken for all pesticides where bees may be exposed. For sprayed products the basis of the risk assessment is the generation of a hazard quotient (HQ) calculated by dividing the application rate of the sprayed product active ingredient by the contact or oral LD50 (whichever is the lower). The Directive then requires under 2.5.2.3: "no authorization shall be granted if the hazard quotients for oral or contact exposure of honeybees are greater than 50, unless it is clearly established through an appropriate risk assessment that under field conditions there are no unacceptable effects on honeybee larvae, honeybee behaviour, or colony survival and development after use of the plant protection product according to the proposed conditions of use".

The result of the calculated hazard quotient is therefore a key decision making point in the risk assessment process in determining if further work is required or the product can be safely used in the presence of foraging honeybees without further evaluation. In the UK, Germany and the Netherlands post-registration monitoring schemes have been established to permit reporting of suspected cases of poisoning of honeybees (incidents) and to inform the regulatory process. This paper reviews the incident data available over the last 25 years from these countries to determine the robustness of the currently used HQ value of 50.

Materials and methods

At the outset of the analysis it was determined that a pesticide incident (i.e. a report from a single use) would be the basis of the comparison with the HQ. An incident often included impacts on more than one colony (in some cases several hundred) but was considered a more robust measure of the impact of a pesticide than the total number of colonies affected which is directly linked to the number present at the time and related to the availability of suitable forage.

Data on the numbers of honeybee poisoning incidents and the pesticide residues detected were collated from countries where there were established monitoring schemes (the UK, the Netherlands and Germany). The details of the national schemes have been reviewed[1-4]. There were no equivalent data available from other European countries. The data available for the UK spanned 1981-2006, for the Netherlands 1989-1998 and 2005-2007 and Germany 1985-2004. These data from the national co-ordination schemes clearly spanned the time period when Directive 91/414 was introduced and therefore provided data for a broad range of pesticides. The incidents were ascribed to pesticides only when residues of the pesticides were detected in bees or, in Germany only, other related materials such as plants on which the bees had been foraging.

There were also differences in national schemes in their approach to pesticide analysis. In Germany, herbicides and fungicides were routinely screened and used as a 'fingerprint' to establish a link between bee and plant material. In the UK, fungicides and herbicides were only screened for when field evidence suggested that they may be implicated. Thus, in Germany multiple pesticides were more likely to be detected in an incident and, whilst a reflection of the relative presence of the pesticides in incident samples, overestimated the true number of incidents directly resulting from its use.

LD50 data for honeybees and application rates were collated and hazard quotients for each pesticide calculated by dividing the application rate (g ai/ha) by the contact and/or oral LD50 (µg ai/bee). In most cases the crop was either not known or was not reported and therefore the application rate used in the HQ was the highest rate for which data were available (liaison.csl.gov.uk). This application rate was unlikely to be significantly different from that actually used at the time although it must be recognised that if rates were higher it may be considered to underestimate the worst-case. The HQ for each pesticide was then compared to the reported number of incidents in which the pesticide was detected.

Results And Discussion

As with any reactionary scheme the incident data were dependent on the willingness of beekeepers to report incidents. It has been well recognised that the willingness of beekeepers to report incidents is affected by their perception of the scheme's/ regulators ability to solve any problems, the need to retain apiary sites and

the effect of any follow-up action on their relationship with landowner/farmer. In both the UK and Germany the reason, where known, for the incident was reported, i.e. normal use, misuse (often caused by misunderstanding of the label, e.g. use on flowering crop) or abuse (deliberate poisoning). However, in many cases, around 70% in 1999-2003 in the UK and up to 41% in Germany, the reason for the incident remained unknown due to problems in identifying both the crop that the bees were foraging on and the particular pesticide application. In the UK deliberate abuse of pesticides accounted for less than 5% of the reported incidents whereas in Germany from 2001-2006 the abuse cases represented up to 32% of the total. There were no reports of pesticide abuse in the Netherlands.

Data were collated (Table 1) for 74 pesticides with a wide range of modes of action. The number of samples in which residues were detected ranged from 1 to 1488 (sulphur). In the UK and Netherlands the number of incidents per year (Figure 1) appeared to be decreasing. For Germany, the graph represents the total number of incidents which were reported to the investigation office by the beekeepers. More than 50% of these incidents were caused by diseases as Varroa, Nosema, virus infections or by deliberate poisoning. The total number of incidents decreased from more than 400 per year at the beginning of the 1980s to less than 100 per year since the middle of the 1990s and remained on this low level except for the year 2003 with 178 incidents (including 645 bee and plant samples).

Table 1 Classes of pesticides detected in incidents

Pesticide class	Number of chemicals	Number incidents/samples	% incidents/samples
Insecticide	43	4250	
OP	21	2075	24.0
Carbamate	7	289	3.3
OC	7	1213	14.0
Pyrethroid	8	673	7.8
Herbicide	3	14	0.2
Fungicide	23	3418	39.5
Veterinary medicines varroacides[1]	4	964	11.1

[1]coumaphos (40%), fluvalinate (18%), benzylbenzoate (13%) and bromopropylate (29%)

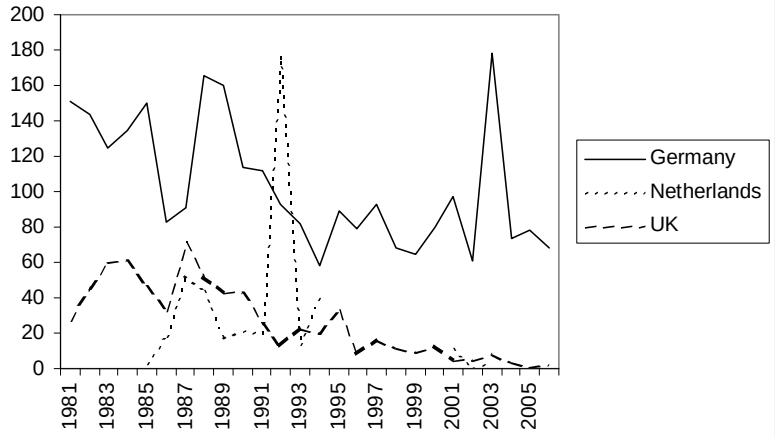

Figure 1 Reported pesticide incidents for the UK and the Netherlands and reported incidents ascribed to pesticides in Germany during the period 1981- 2006

Insecticides were detected in 49% of the samples with fungicides detected in 40%. Of the insecticides organophosphorus compounds contributed to 49% and organochlorines 29%. The major organophosphorus pesticide residues were dimethoate (23%); parathion (17%); triazophos (14%); methyl parathion (14%) and phosalone (13%). The major contributor to the organochlorine related residues was gamma HCH (53%) and in the pyrethroid class the major contributors were cypermethrin (42%); l-cyhalothrin (25%) and alphacypermethrin (16%). Of the carbamate residues detected 53% were fenoxycarb (an IGR which is relatively non-toxic to adult bees but results in brood mortality); 19% were bendiocarb and 14% were carbaryl. Carbaryl was banned in Germany in 1982, as it had caused the serious losses of honeybee populations in German vine growing areas and was involved in more than 50 % of the incidents at that time

The most frequently detected pesticides in incidents reported in each country were sulphur, lindane/gamma HCH, vinclozolin and coumaphos (Germany); dimethoate, parathion and methyl parathion (the Netherlands) and triazophos, dimethoate. lindane/gamma HCH and bendiocarb in the UK. Of these sulphur was used as fertiliser and fungicide in agriculture and against the waxmoth in apiculture. Coumaphos was used as a varroacide by beekeepers. Lindane residues may derive from wax from non-EU countries where it is still used in agriculture. In Germany it was banned in 1977 (West) and 1990 (East), respectively. Vinclozolin is a fungicide of low toxicity to honeybees (>200 µg /bee) that may be present in incidents with other pesticides.

Bendiocarb contributed more than 50% of the reports related to carbamate insecticide use and all were in the UK. Use in the UK is restricted to the treatment of feral bee and wasp nests and poor sealing of the treated colonies is known to lead to robbing and mortality at colonies in the vicinity.

The number of incidents reported were compared with the calculated hazard quotient (HQ) values and shown in Figure 2. The low correlation coefficient (r^2=0.03) showed there was no linear relationship between the HQ and the number of reported incidents but it is the threshold value of 50 that is of importance in this context. There were two pesticides which were detected in over 100 incidents but had HQ values below 50. These were captan with an oral LD50 of 91 µg ai/bee and application rate of 2900 g ai/ha (HQ 32) and fluvalinate with an LD50 of 4.8 µg ai/bee and application rate of 48 g ai/ha (HQ=10). All the other pesticides with HQs lower than 50 (9) showed less than 100 reported incidents over the 25-year period in all three countries.

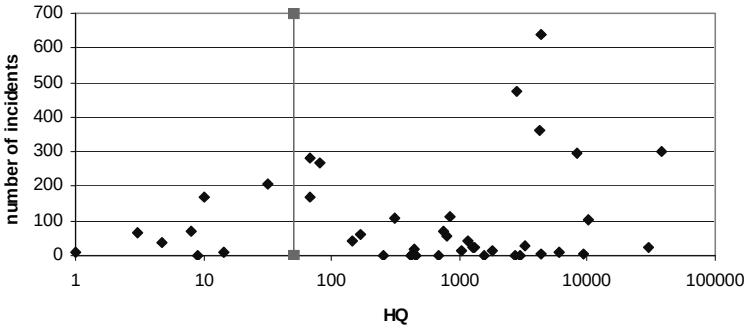

Figure 2 Comparison of the total number of reported incidents involving pesticides and the hazard quotient (HQ) for each pesticide (R^2 =0.03)

The high number of samples reported with fluvalinate residues (169 samples in Germany) was related to its use as a varroacide rather than as an insecticide. Fluvalinate residues have been regularly reported in the UK but residues were very low and not attributed to pesticide poisoning[1]. If the incidents were caused by the agricultural formulation such as Mavrik which is applied at 48 g ai/ha and with an LD50 of 4.8 µg ai/bee then exposure of the bee to over 17 µl of the applied product would have caused mortality. This was

compared with a pesticide known to cause incidents - dimethoate, application rate 336 g ai/ha, volume 200 l/ha and LD50 0.12 µg/bee - 0.007 µl would have resulted in mortality.

Captan was a widely used phthalimide fungicide and therefore it is likely that as with the wide range of fungicides reported in samples (e.g. sulphur, vinclozolin) it purely demonstrated exposure. Based on an application rate of 2900 g/ha and an application volume of 200 l/ha this was equivalent to 14.5 g/ l or 14.5 µg/µl. With an LD50 of 91 µg ai/bee a bee would need to be exposed to 6.2 µl of the applied product to result in mortality. In some cases, however, fungicide exposure may have increased the toxicity of other pesticides present, e.g. the pyrethroids. The azole fungicides are of particular concern in this regard and were reported in a total of 311 samples (9% of the fungicides reported). Of these 51 were directly attributable to the tank mixing of pyrethroids and EBI fungicides[3] and the 3 UK alphacypermethrin incidents and a single deltamethrin incident between 1994-2003 also contained fungicides[1].

Use of dimethoate on field beans and triazophos and gamma-HCH on oilseed rape were reported as the major causes of incidents and had high HQ values. The use of dimethoate on oilseed rape was withdrawn in the UK in 2000. All products using gamma-HCH were withdrawn in the UK in 2001 with all uses ceasing in 2003. In the Netherlands the use of dimethoate and parathion on potato crops to control aphids resulted in mortality due to bees foraging on aphid honeydew or flowering weeds within the crop. A similar issue related to bees foraging on aphid honeydew and flowers (including weeds) in potato crops occurred in Germany due to the misuse of bee hazardous products containing dimethoate, parathion, chlorpyrifos, cypermethrin or methamidophos. In these cases incidents occurred when bees were exposed to spray coating covering the honeydew which had become wet due to the humidity in the air the morning after the application. As a consequence the aphid control thresholds in potatoes were reduced and a publicity campaign was undertaken with farmers. In 2006 during similar weather conditions only 17 incidents in potatoes were reported instead of 119 incidents ascribed to potatoes in 2003.

The presence of several pesticides within a sample doesn't necessarily implicate all the pesticides in the incident but does demonstrate that bees may be exposed to a range of compounds whilst foraging on treated crops. In Germany up to 5 different pesticides were detected in samples of honeybees and up to 3 in plant samples[4]. In the UK pesticide mixtures were found in 6% of incidents where multiple residue detection was undertaken between 1981 and 1991 and 4% between 1994 and 2003.

The high hazard quotient of the pyrethoid insecticides would suggest that incidents would occur in the field due to their widescale use and a significant number of incidents have been reported. However, field studies have widely demonstrated that repellency to the pyrethroid applications occurs on treated crops and therefore far lower mortality than may be expected occurs in the field[5]). For some pyrethroid insecticides applied at low rates it has been demonstrated in large-scale field studies that when applied alone during bee flight no increased mortality or other impact on honey bee colonies occurred. The number of incidents may be due to application of tank mixes of pyrethroids with EBI fungicides during times when bees are actively foraging on the crop (the label recommends application early morning or late evening). Of the 11 incidents between 1994 and 2003 half contained residues of pyrethroid insecticides and fungicides. The EBI fungicides increase the toxicity of pyrethroid insecticides by blocking their cytochrome P450 dependent metabolism[6]. The increased toxicity of the combination may result in exposure to a toxic dose before repellency can become effective thus increasing the risk to the bees[7]. In Germany the number of incidents reported has resulted in labelling that either prohibits the use of mixing of pyrethroids and EBI fungicides generally during flowering or allows application during flowering but only in the evening after bee flight[3].

Conclusions

This review of incidents in Europe over the last 25 years suggests that the HQ approach to risk assessment for honeybees offers an appropriate level of protection. The incident schemes have been invaluable in identifying agronomic practices resulting in honeybee mortality and changes have been made to labelling to address such issues, e.g. not using tank mixes of pyrethroids and EBI fungicides during bee flight, ensuring absence of flowering weeds in non-flowering crops, spraying early morning or late evening. Without these

incident schemes honeybee incidents are likely to go undetected or the cause unproven. The decrease in the numbers of incidents reported supports the ascertain that such scheme positively contribute to the regulatory process and can provide confidence in the risk assessment approaches.

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Periodical honey bee colony losses in Germany: preliminary results from a four years monitoring project

Martina Janke[1*], Peter Rosenkranz[2], Working group of German bee institutes[3]

[1] LAVES Institut für Bienenkunde Celle, Herzogin-Eleonore-Allee 5, D-29221 Celle, Germany
[2] Universität Hohenheim, Landesanstalt für Bienenkunde, August-von-Hartmann Straße 13, 70599 Stuttgart
[3] http://www.ag-bienenforschung.de
*phone: 0049(0)51419050356, fax: 0049(0)51419050344, e-mail : martina.janke@laves.niedersachsen.de

Abstract

Within the framework of the German Bee Monitoring Project winter losses of bee colonies were evaluated from the database of 120 beekeepers and 1200 bee colonies by assessing the following parameters: data on the apiary (site, nuclei, movement of colonies, *Varroa* treatment), strength of the colonies in autumn and spring, honey yields, residues in bee bread (stored pollen), bee disease analysis.

During the last four years the winter losses of the monitoring beekeepers were between 8 and 16% and showed regional differences. The loss rates were clearly lower than those of non-monitoring beekeepers.

In 215 bee bread samples analysed with a sensitive multi-method, more than 55 active ingredients were found. Most active ingredients were found in traces but often in combinations. Primarily fungicides, varroacides and herbicides were found. Clothianidin was not found in any sample. Imidacloprid was found in one sample at the limit of detection.

4400 data sets were statistically analysed for the identification of triggers with negative influence on overwintering. The winter losses were significantly correlated with *Varroa* infestations and virus infections in autumn. It was concluded that no acute effects on honey bees have to be expected on the basis of the evaluated residue data. For testing potential sublethal or long term effects a useful test design has to be developed. The project will be continued in 2009.

Keywords: German Bee Monitoring Project, colony losses, *Apis mellifera, overwintering*

Introduction

The German Bee Monitoring Project was established in 2004 with the aim of finding explanations for periodical colony losses. It was considered advisable to involve all people dealing with bees and apiaries for this long term and large project with scientific approach and standing in the focus of the press and public political discussions. The founded project council consisted of national beekeeper associations, farmer association, authorities, German apicultural institutes and chemical industry. Financial support was given by the chemical industry on a level of nearly 50%.

The project cooperation partners planned the project. For collecting data of bee colonies it was decided to work on a large basis. A unique structure was established for assessing the health status of colonies effectively and scientifically. This is the first and only long-term monitoring project in the world providing verified data.

Data assessments

Data on the development of 1200 bee colonies in 120 apiaries spread all over Germany were assessed over four years by standardized methods. More than 100.000 data were assessed and about 5200 statistically analysable records of colonies were created. The participating apiaries represent the whole\ German spectrum in size, beekeeping management and use of honey flow. The beekeepers provided general data about their apiaries (all colonies, which means about 7000 colonies in total). Data on the apiary, site of the apiary (climate, honey flow, plant protection measures), colony losses, honey yields, beekeeping practice (movement of colonies, *Varroa* treatment, nuclei) were assessed per season.

The beekeepers, with a supervisor of the responsible bee institute, focussed on details of the 10 monitored colonies such as the population dynamics (strength and the development of the colonies before and after overwintering) and samplings (bee samples for diseases) three times per season, honey samples two times per season, one bee bread sample.

The collected bee samples were analysed in the laboratory for *Varroa* infestations, virus infections (ABPV - Acute Bee Paralysis Virus, DWV - Deformed Wing Virus, KBV - Kashmere Bee Virus, SBV - Sacbrood Bee Virus, IAPV - Israel Acute Paralysis Virus since 2007), *Nosema* sp. infection, *Malpighamoeba mellificae* infection and *Acarapis woodi* infestation. The collected honey samples were analysed for their botanical origin. The bee bread samples were analysed for residues with a multi-method detecting 258 active ingredients by an independent laboratory (LUFA institute, Speyer). All data were saved in a central database.

Results and conclusions

The most important results were summed up by the German apiculture research institutes in annual interim reports. The following results are part of the interim report 2004 to 2008[1].

Colony losses

The average winter losses were lower than those of the disaster year 2002/2003 with a loss rate of 28.9%. Noticeable are the annual and regional differences. Among them quite high losses occurred (table 1a). In single cases high losses occurred up to total losses (table 1b). Over 10% of the monitoring beekeepers had no losses over the four project years. The loss rates of the monitoring beekeepers were about 50% lower than those of non-monitoring beekeepers as it appeared from surveys over the project years. Maybe that the

monitoring beekeepers represent a "positive selection" and were better supervised. It was concluded that good management has a big influence on the bee health.

Table 1a Overwintering losses (in %) of monitored colonies. Over 7000 colonies were monitored by beekeepers, supervised by the bee institutes

Supervising bee institute	**2004/2005**	**2005/2006**	**2006/2007**	**2007/2008**
Number of colonies before overwintering (n)	**7240**	**7168**	**7013**	**7187**
Celle	2,7	4,0	18,5	7,6
Freiburg	12,0	14,0	15,9	18,5
Halle	11,6	13,6	7,2	36,5
Hohenheim	6,3	2,2	1,4	1,8
Hohen-Neuendorf	9,0	24,8	3,1	17,8
Kirchhain	7,1	13,9	12,0	15,1
Mayen	5,2	12,1	6,1	16,9
Münster	5,7	14,1	0,4	14,0
Veitshöchheim	11,5	16,2	15,0	14,6
Total	**7,9** [a)]	**12,8** [a)]	**8,8** [a)]	**15,9** [a)]
	6,6 [b)]	**13,1** [b)]	**11,0** [b)]	**12,8** [b)]

[a)] Average percentage of losses; [b)] Losses calculated over the total number of colonies

Table 1b Winter loss levels of participating apiaries. Of all apiaries participating during the four project years nearly 1/3 had no losses while about 15% had losses over 20%.

Level of losses [%]	**Apiaries [number]**	**Apiaries [%]**
0	156	32,8
0-10	157	33,0
10-20	89	18,7
20-30	31	6,5
30-40	15	3,2
40-50	13	2,7
50-60	3	0,6
60-70	1	0,2
70-80	5	1,1
80-90	2	0,4
90-100	4	0,8

Honey yields

The reported project years were good up to very good years of honey yields with almost more than one honey flow during the seasons (table 2).

Table 2 Average honey yield per participating colony in kg/colony

Supervising bee institute	2004/2005	2005/2006	2006/2007	2007/2008
Celle	41,7	41,0	40,3	40,2
Freiburg	28,6	66,3	87,5	-
Halle	38,9	49,5	49,5	37,5
Hohenheim	32,8	57,3	34,3	21,3
Hohen-Neuendorf	37,9	55,8	50,9	51,8
Kirchhain	44,6	44,3	40,2	39,9
Mayen	43,5	38,3	41,0	37,8
Münster	49,4	45,6	38,7	16,4
Veitshöchheim	37,9	42,7	34,7	24,5
Total	**39,5**	**49,0**	**46,3**	**33,7**

Bee diseases

Varroa

As one of the most important criteria the *Varroa* infestation level was assessed at the start of winter (after late summer treatment).

The *Varroa* infestation before winter varied between the supervising institutes and years of monitoring. During the first project years the average was under 5%. In 2007, the year of high *Varroa* infestations, the average of 6% was clearly higher (table 3). As the high *Varroa* infestation was widespread, all bee institutes warned in good time. Probably the warnings for a consequent treatment were better put into effect by the participating beekeepers than by others.

Table 3 Average level of *Varroa* infestation (in %).in adult bees of the participating bee colonies in October at the start of winter

Supervising bee institute	2005/2006	2006/2007	2007/2008
Celle	2,6	4,2	3,3
Freiburg	2,0	6,4	11,0
Halle	9,1	5,5	11,7
Hohenheim	2,4	3,6	2,5
Hohen-Neuendorf	7,1	3,3	4,5
Kirchhain	8,7	5,9	4,8
Mayen	3,2	2,9	4,0
Münster	Bottom board diagnosis	Bottom board diagnosis	7,8
Veitshöchheim	3,6	5,5	4,3
Total	**4,8**	**4,7**	**6,0**

The damages caused by *Varroa* were limited. An infestation level of 6% means an average of 600 mites per colony if the strength of the colony is 10.000 bees at the start of winter. The absolute damage threshold is at 10%. The infestation levels found in the monitored colonies show that some of the colonies exceeded this threshold. Some high losses with individual participating beekeepers could be related to delayed or insufficient *Varroa* treatment.

Bee viruses

Depending on the project year and the virus type the percentage of positive samples was between 6% and 33% (table 4). Noticeable is the high level of positive DWV samples in autumn 2007. The high *Varroa* infestation levels found in 2007 are probably relevant. Surprisingly, the occurrence of viruses was very different between the German regions. The KBV was only found in two samples during the whole monitoring period. It is worth mentioning that only bee heads were analysed. This leads to less positive results than by analysing whole bees

Table 4 Average viruses infection levels (in % of analysed samples) in autumn bee samples. Not all samples were analysed because of high costs. The number of analysed samples is given in the table.

	Acute Paralyse Virus (ABPV) in %				**Sacbrood Virus (SBV) in %**			
Institute	**2004/05**	**2005/06**	**2006/07**	**2007/08**	**2004/05**	**2005/06**	**2006/07**	**2007/08**
Celle	4,2	33,3	19,5	2,6	0	13,9	17,1	5,1
Freiburg	20,0	7,4	0,0	0,0	0,0	22,2	14,8	18,5
Halle	18,2	40,0	13,3	16,7	13,6	0,0	0,0	5,6
Hohenheim	0,0	0,0	2,2	22,2	33,3	37,8	-	28,9
Hoh-Neuendorf	2,9	8,5	0,0	3,0	1,4	0,0	0,0	1,5
Kirchhain	16,9	21,2	22,2	30,3	16,9	6,1	11,1	6,1
Mayen	0,0	30,3	8,3	22,2	20,0	9,1	8,3	8,3
Münster	0,0	0,0	0,0	23,3	0,0	0,0	0,0	6,7
Veitshöchheim	0,0	0,0	0,0	0,0	12,1	1,4	8,1	0,0
Total	**6,4**	**12,1**	**6,1**	**11,1**	**12,4**	**9,1**	**7,6**	**7,9**

	Deformed Wing Virus (DWV) in %				**Number of analysed samples n**			
Institute	**2004/05**	**2005/06**	**2006/07**	**2007/08**	**2004/05**	**2005/06**	**2006/07**	**2007/08**
Celle	8,3	38,9	56,1	33,3	24	36	41	39
Freiburg	0,0	22,2	14,8	37,0	15	27	27	27
Halle	18,2	60,0	26,7	44,4	22	15	15	18
Hohenheim	2,4	0,0	2,2	66,7	42	45	45	45
Hoh-Neuendorf	21,7	14,0	23,2	25,8	69	94	69	66
Kirchhain	4,2	21,2	25,0	33,3	71	33	36	33
Mayen	0,0	18,2	5,6	52,8	30	33	36	36
Münster	0,0	0,0	6,3	23,3	15	18	16	30
Veitshöchheim	0,0	0,0	9,5	8,0	58	72	74	75
Total	**7,2**	**14,8**	**18,7**	**32,8**	**346**	**373**	**359**	**369**

	Israel Acute Paralysis Virus (IABPV) in % (only 2007)			
Institute	**Negative**	**Positive**	**Uncertain**	**Total**
Celle	87,2		12,8	39
Halle	100			18
Hohenheim	95,5		4,5	44
Hoh-Neuendorf	100			66
Kirchhain	100			33
Mayen	100			36
Münster	100			30
Veitshöchheim	100			75
Total	**97,7**	**0,0**	**2,4**	**341**

***Nosema* sp.**

During the first two years one third of the analysed spring samples were positive for *Nosema* sp., but only 8 % showed a high infection level (table 5). Surprisingly, during the third year the amount of positive samples was below 20%. Analysis showed that most samples were infected with *Nosema ceranae*. Noticeable is the increase of *Nosema* sp. infections in spring 2008. Remarkable is that in summer 2008 samples the percentage of positive findings was still 25%.

Table 5 Average *Nosema* sp. infection levels in spring (in % of analysed samples; n = number of analysed samples). In 2007/2008 autumn and summer samples were also analysed.

	Spring 2005					Spring 2006				
Institute	**no**	**low**	**medium**	**high**	**n**	**no**	**low**	**medium**	**high**	**n**
Celle	78,8	6,1	11,1	4,0	99	61,4	2,9	27,9	7,9	140
Freiburg	76,0	10,0	12,0	2,0	50	33,3	6,2	33,3	27,2	81
Halle	10,0	70,0	15,0	5,0	40	52,9	44,1	2,9	0,0	34
Hohenheim	42,8	15,2	27,5	14,5	138	66,0	27,9	0,7	5,4	147
Hoh-Neuendorf	75,8	4,3	7,4	12,6	231	70,5	11,0	10,1	8,4	227
Kirchhain	63,0	16,8	11,8	8,4	119	76,0	5,2	1,0	17,7	96
Mayen	74,8	16,8	6,5	1,9	107	56,8	21,2	15,3	6,8	118
Münster	86,0	10,0	4,0	0,0	50	92,7	5,5	1,8	0,0	55
Veitshöchheim	72,8	15,1	9,6	2,5	239	28,2	51,7	15,4	4,6	259
Total	**67,7**	**13,9**	**11,6**	**6,9**	**1073**	**56,3**	**22,2**	**13,1**	**8,5**	**1159**

	Spring 2007					Autumn 2007				
Institute	**no**	**low**	**medium**	**high**	**n**	**no**	**low**	**medium**	**high**	**n**
Celle	86,0	2,3	10,1	1,6	129	81,5	9,2	7,7	1,5	130
Freiburg	79,4	15,7	4,9	0,0	102					
Halle	72,3	27,7	0,0	0,0	47	98,2	1,8	0,0	0,0	57
Hohenheim	83,3	11,9	4,8	0,0	126	70,0	22,7	7,3	0,0	150
Hoh-Neuendorf	75,4	7,0	8,3	9,2	228	93,2	2,3	2,7	1,8	219
Kirchhain	91,8	1,0	5,2	2,1	97	97,3	0,0	0,9	1,8	110
Mayen	88,8	7,5	3,7	0,0	107	90,8	2,5	1,7	5,0	120
Münster	93,5	6,5	0,0	0,0	62	98,4	0,0	0,0	1,6	61
Veitshöchheim	81,2	13,7	2,4	2,7	255	80,2	6,7	8,7	4,0	252
Total	**82,6**	**9,6**	**5,0**	**2,8**	**1153**	**86,4**	**6,6**	**4,7**	**2,3**	**1099**

	Spring 2008					Summer 2008				
Institute	**no**	**low**	**medium**	**high**	**n**	**no**	**low**	**medium**	**high**	**n**
Celle	62,6	22,0	12,2	3,3	123					
Freiburg										
Halle	50,0	11,8	8,8	29,4	34					
Hohenheim	38,7	50,0	5,3	6,0	150					
Hoh-Neuendorf	64,4	8,7	7,2	19,7	208					
Kirchhain	85,7	4,4	4,4	5,5	91					
Mayen	68,9	6,7	6,7	17,6	119	86,1	8,9	5,0	0,0	101
Münster	73,9	8,7	2,2	15,2	46	86,7	6,7	6,7	0,0	30
Veitshöchheim	58,2	28,9	10,0	2,8	249	69,2	24,7	6,1	0,0	247
Total	**61,3**	**20,8**	**7,7**	**10,2**	**1020**	**75,1**	**19,0**	**5,8**	**0**	**378**

Malpighamoeba mellificae

The total amount of positive samples is low. In southern Germany more infections were found (table 6).

Table 6 Average *Malpighamoeba mellificae* infection levels in spring (in % of analysed samples; n = number of analysed samples). In 2007/2008 autumn and summer samples were also analysed.

	Spring 2006					Spring 2007				
Institute	**no**	**low**	**medium**	**high**	**n**	**no**	**low**	**medium**	**high**	**n**
Celle	96,4	3,6	0,0	0,0	140	100,0	0,0	0,0	0,0	129
Freiburg	-	-	-	-	-	0,0	97,1	2,9	0,0	102
Halle	97,1	2,9	0,0	0,0	34	100,0	0,0	0,0	0,0	47
Hohenheim	16,3	66,7	15,6	1,4	147	50,0	46,8	3,2	0,0	126
Hoh-Neuendorf	100,0	0,0	0,0	0,0	227	100,0	0,0	0,0	0,0	228
Kirchhain	100,0	0,0	0,0	0,0	96	100,0	0,0	0,0	0,0	96
Mayen	86,3	12,8	0,9	0,0	117	100,0	0,0	0,0	0,0	109
Münster	100,0	0,0	0,0	0,0	54	-	-	-	-	-
Veitshöchheim	-	-	-	-	-	54,2	45,8	0,0	0,0	236
Total	**82,2**	**14,6**	**2,9**	**0,2**	**815**	**74,6**	**24,8**	**0,7**	**0,0**	**1073**

	Autumn 2007					Spring 2008				
Institute	**no**	**low**	**medium**	**high**	**n**	**no**	**low**	**medium**	**high**	**n**
Celle	99,2	0,8	0,0	0,0	130	71,5	28,5	0,0	0,0	123
Freiburg										
Halle	91,2	8,8	0,0	0,0	57	100,0	0,0	0,0	0,0	34
Hohenheim	100,0	0,0	0,0	0,0	150	88,0	12,0	0,0	0,0	150
Hoh-Neuendorf	100,0	0,0	0,0	0,0	219	100,0	0,0	0,0	0,0	209
Kirchhain	100,0	0,0	0,0	0,0	110	100,0	0,0	0,0	0,0	91
Mayen	100,0	0,0	0,0	0,0	103	98,3	1,7	0,0	0,0	119
Münster	100,0	0,0	0,0	0,0	61	100,0	0,0	0,0	0,0	46
Veitshöchheim	87,3	12,7	0,0	0,0	252	87,3	12,7	0,0	0,0	251
Total	**96**	**4**	**0**	**0**	**1082**	**91**	**9**	**0**	**0**	**1023**

	Summer 2008				
Institute	**no**	**low**	**medium**	**high**	**n**
Celle					
Freiburg					
Halle					
Hohenheim					
Hoh-Neuendorf					
Kirchhain					
Mayen	84,9	15,1	0,0	0,0	119
Münster	100,0	0,0	0,0	0,0	30
Veitshöchheim	85,4	14,6	0,0	0,0	247
Total	**86**	**9**	**0**	**0**	**396**

Residue analysis/residues in bee bread (stored pollen)

In the first project year honey and bee bread samples were specifically analysed for imidacloprid residues of treated oilseed rape sites. In 36 nectar/honey samples no residues were found and in only two out of 48 pollen/bee bread samples residues were found at the limit of quantification (1 ppb).

Bee bread samples were analysed for assessing the basic residue contamination of the colonies. Higher amounts of active ingredients can be expected in pollen than in nectar. Bee bread is consumed by nurse bees and larvae over a longer period which may result in long term effects.

First a method for detecting all relevant active ingredients had to be established. The LUFA in Speyer developed a sensitive multi-method for detecting and quantifying 258 active ingredients in bee bread samples. The limits of quantification are between 3 and 10 in single cases 15 µg/kg bee bread. Thus 215 bee bread samples of 2005 to 2007 were analysed. Only samples collected during or after the flowering of oilseed rape in spring were analysed because this crop is intensively treated with plant protection products and oilseed rape pollen and nectar are very attractive for bees.

In the first test series of 2005 and 2006, 105 bee bread samples from colonies exposed to oilseed rape and showing negative overwintering success were analysed. Here 42 active ingredients with a number of 1 to 46 positive detections were found (figure 1).

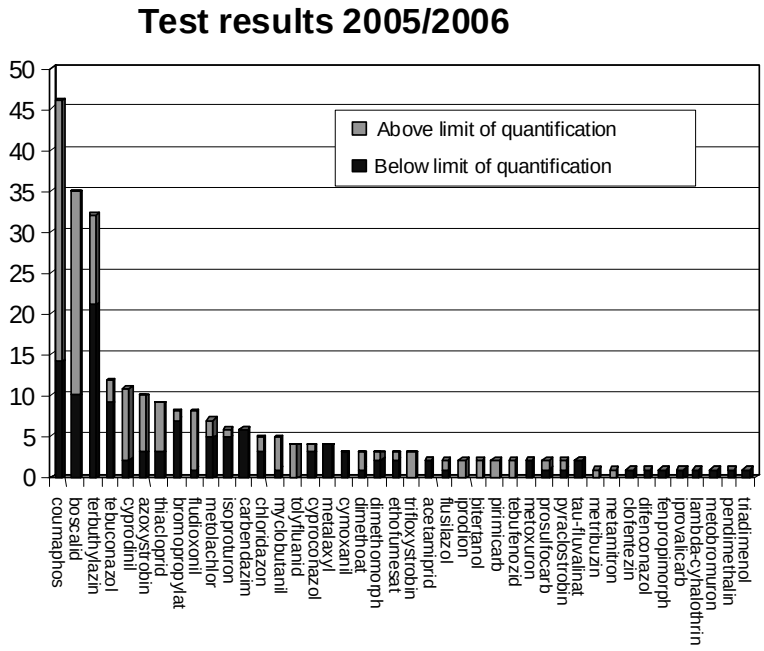

Figure 1 Frequency of positive detections of active ingredients found in 105 bee bread samples of spring 2005 and 2006.

In nearly all samples more than one active ingredient was detected. Only in 25 samples (24%) no residues were found. Coumaphos (46 positive results, varroacide), boscalid (35 detections, fungicide) and terbuthylazin (32 positive results, herbicide) were found most often. And thiacloprid is the insecticide which was found most often (9 positive results, max. 199 µg/kg). Other detected insecticides were dimethoate (3 positive results), azetamiprid (2 positive results), pirimicarb (2 positive results), tau-fluvalinat (2 positive

results) and lambda-cyhalotrine (1 positive result). The amounts of the 5 insecticides were below 10 µg/kg (except dimethoate, 20 µg/kg). Apart of these single results the detected amounts were small: 112 out of 171 positive results were below 10 µg/kg. Imidacloprid was not found in any sample.

In the project year 2007/2008, 110 additional bee bread samples of the season 2007 were analysed. Aliquots of the extracted samples were analysed by BayerCropscience for neonicotinoids. Numbers and quantities of the residues are similar to the results of the years 2005/2006. Again 42 active ingredients were found with a number of 1 to 67 positive detections almost in traces (figure 2). In comparison with the previous years single active ingredients were not found any longer while others were found for the first time. The frequency was different: the active ingredient coumaphos was in 4th position (33 positive results, varroacide). The number of positive samples increased for the active ingredients boscalid (67 positive results, fungicide), thiacloprid (62 positive results, insecticide, classified in Germany as not harmful for bees), and terbuthylazin (48 positive results, herbicide).

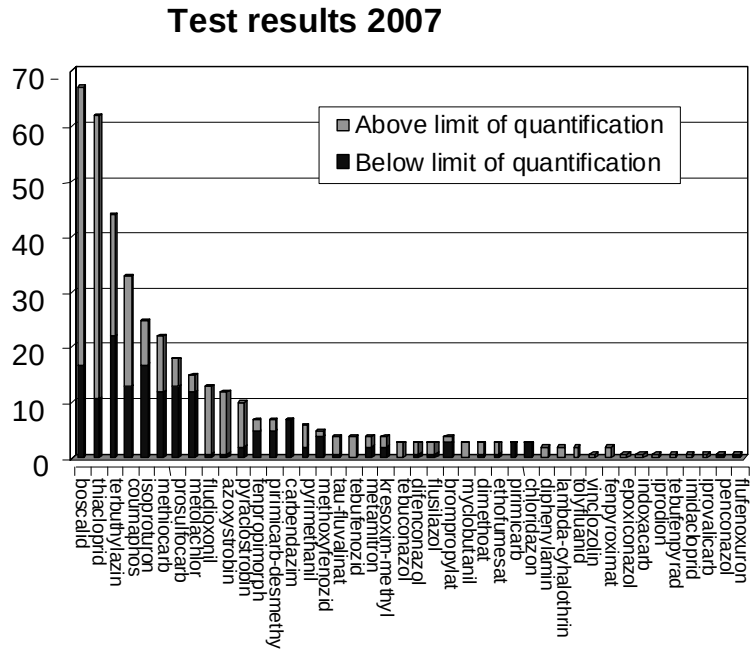

Figure 2 Frequency of positive detections of active ingredients found in 110 bee bread samples of spring 2007.

Of special interest were the active ingredients of the neonicotinoid group, which are classified in Germany as harmful for bees. In 215 analysed samples of 2005 to 2007 clothianidin was not found in any sample while imidacloprid was found in one sample (3 µg/kg).

Preliminary conclusions:

- The results of the residue analysis represent the first evaluations of residue contamination of bee bread in Germany and give important basic data for further evaluations;
- No residues of active ingredients classified as harmful for bees of which acute side effects for bees can be expected were found in bee bread. The same applied for neonicotinoids which were not found in spring samples either with just one exception.

- A considerable contamination with active ingredients was found in the bee bread samples. It is uncertain if this contamination with almost more than one active ingredient per sample will have negative long-term effects on colonies (bee brood, nurse bees).
- For evaluating the effects of the residue contamination on the development and overwintering success of a colony, specific and long term assessments have to be run with colonies being treated with different residue amounts.
- For testing potential sublethal or long term effects a useful test design has to be developed yet.
- Coumaphos should be replaced by other active ingredients in *Varroa* treatments.

Statistical evaluations

In a first step it was statistically analysed if certain parameters (site of the apiary, bee diseases, beekeeping management) were significantly correlated with colony losses or bad overwintering results. Nearly 4400 data sets were statistically analysed with non-parametric tests (U-test, Chi^2-test). Various parameters were tested for significance of differences between surviving colonies and colonies that died. In evaluations still going on the data of different project years and different parameters will be linked. The results will be published in the following months.

During the four years of the project the loss rates were below the threshold of the disaster in 2002/2003. Besides losses also a comparisons of the bee population before and after winter were recorded. This offers the possibility to evaluate sublethal effects which potentially weakened colonies during winter. Factors with negative influence tendencies on wintering and factors to be excluded as triggers could be identified.

Based on the current evaluations it was concluded:

1. Between oilseed rape sites and non-oilseed rape sites no differences were found for colony losses neither for the overwintering quotient (= colony strength in autumn divided by colony strength in spring). The evaluations are based on 2325 data sets of the project years 2005/2006 and 2006/2007. The results indicate even better overwintering success for colonies exposed to oilseed rape sites.
2. Highly significant correlations were found between winter losses and the *Varroa* infestation levels in autumn. The risk for colony loss increases with the number of mites in the colony in autumn.
3. Similarly the correlations between the infection with ABPV and DWV in autumn and winter losses were significant.
4. No significant correlations were found for *Nosema* sp. infections.
5. Surprisingly, the age of the queen was significantly correlated with the winter losses. Young queens were more successful. Not surprisingly, the strength of the colony in autumn is significantly correlated with the winter losses. The risk of winter losses decreases with the strength of the colony in autumn.
6. No significant effects were found for the type of syrup used for feeding before wintering, for the type of boxes (wood/plastic), for the size of frames or young colony/old colony.

The annual interim reports of the project are published on http://www.ag-bienenforschung.de. A detailed report will be published by the German apiculture research institutes in spring 2009.

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Bee poisoning incidents in Germany in spring 2008 caused by abrasion of active substance from treated seeds during sowing of maize

Pistorius, J.[1*], Bischoff, G.[2], Heimbach, U.[1], Stähler, M.[2]

Julius Kühn-Institut:
[1] Institute for Plant Protection in Field Crops and Grassland,
[2] Institute for Ecological Chemistry, Plant Analysis and Stored Products Protection
*Tel.: +49 531 299 4525; Fax: +49 531 299 3008. E-mail: jens.pistorius@jki.bund.de

Abstract

In spring 2008 a high number of bee poisoning incidents was recorded during sowing of maize in the Upper Rhine valley and in South Bavaria near Passau. More than 11.500 honey bee colonies from about 700 beekeepers in the Upper Rhine valley showed symptoms of insecticide poisoning. The reason for the poisoning was the abrasion of dust from maize seeds treated with the insecticide Poncho Pro (a.s. clothianidin) during the sowing process and blowing out of this dust containing the active substance into the environment with pneumatic sowing machines, resulting in contamination of nectar and pollen. The poisonings occurred in areas in southern Germany in which an eradication program for the quarantine pest *Diabrotica virgifera virgifera* was active and where clothianidin was used at a high rate (125 g a.s. /ha) on a large scale.

An exceptionally high amount of dust of up to 80 g per 100.000 kernels of maize was detected in some of the maize seed batches. The chemical analysis of dust, plant samples, bee samples, fresh pollen and bee bread confirmed the poisoning by clothianidin originating from treated maize seeds. No correlation with any bee pathogens was detected.

Keywords: seed treatment, drilling machines, neonicotinoid, clothianidin, dust, maize, drift, bee poisoning, honey bees

Introduction

A high density of bee colonies in Germany is located in the Upper Rhine valley in Baden-Württemberg, due to the mild climate which promotes the overwintering of the colonies and a rapid development of the colonies in spring. Furthermore a high amount of bee attractive crops such as fruit trees and winter oilseed rape with short distances to other foraging possibilities like sweet chestnut, white fir and Norway spruce allow for good honey yield and excellent bee keeping conditions. At the beginning of April 2008, above-average overwintering losses of bee colonies of up to about 40% in some areas had been registered in the region, already before the first incidents of poisonings were reported in the last week of April 2008 during spring development of the colonies. High numbers of bee poisoning incidents were recorded during sowing of maize in the Upper Rhine valley and in parts of South Bavaria.

First reports of bee poisonings in a single municipality in the upper Rhine valley at end of April 2008 were followed within a few days by reports of some hundred beekeepers claiming bee damages, describing typical clinical symptoms of acute insecticide poisoning. Dying bees with obvious symptoms of intoxication like cramping, disoriented behaviour and abnormal wing movements were discovered in front of the hives and inside the hives, amounting up to several thousands of dead bees per day. Mortality remained at a higher level up to several weeks, resulting in weakened colonies. Some colonies showed only minor bee losses and only a slightly enhanced mortality, while other colonies showed severe damages; the scale of impact of the poisoning of colonies damage varied between 10-90%. Total losses of colonies were reported in only a few cases. Damages were not equally distributed in all local communities of the area; in some communities no bee poisonings were noticed. Since poisoning incidents happened on a large scale, after few days a temporal and spatial connection with the sowing of clothianidin treated maize was suspected and soon confirmed by residue analyses of the samples from incidents.

In the areas with bee poisonings maize seeds were treated with clothianidin for eradication of the western corn rootworm (*Diabrotica virgifera virgifera*), which is, according to the Directive 2000/29/ EG, classified as a quarantine pest by the EU. Compared to the normal use rate for wireworm control on about 5 % of the maize growing area in Germany (50 g a.s. clothianidin/ha), a high rate of clothianidin (125 g a.s./ ha) was used for the eradication purpose on large scale in the affected regions of southern Germany. The seed treatment Poncho Pro with the active substance clothianidin at the high rate had already been used in Germany on a smaller scale in 2006 and 2007, but no bee poisoning incidents had been reported. In 2008, although Poncho Pro dressed maize seeds were used in a larger area in the upper Rhine valley for eradication of *Diabrotica virgifera virgifera*, only some communities reported a high number, but other only a very low number or no bee poisoning incidents at all.

Material and Methods

Sampling, reporting and documentation of poisoning incidents

First samples of bees and plants were taken by the affected bee keepers. Samples were sent to the federal examination centre for bee poisoning incidents at the Institute for Plant Protection in Field Crops and Grassland, Julius Kühn-Institut (JKI).

As within a few days the extent of damage on a large scale became obvious, further sampling of bee and plant samples, documentation and initial collection of claims and reports were conducted by specialized local consultants for bee-keeping, experts from the beekeepers associations, departments from the Ministry of Food and Rural Land (MLR) and the Regional Councils of Stuttgart and Freiburg (RPS, RPF) and the plant protection services of Baden-Württemberg in cooperation with the beekeepers. Additional to residue analyses of bees, bee matrices and plants by the JKI also the state laboratory in Baden-Württemberg, Landwirtschaftliches Technologiezentrum, Augustenberg (LTZ) and a laboratory of Bayer CropScience carried out residue analyses. The consultants for bee-keeping or experts from the bee keepers association examined the colonies in order to estimate the damage and the extent of damage of the colonies.

Analyses of plant and bee samples

Bee samples were screened for common bee parasites, like *Varroa, Nosema*, and amoeba. A visual inspection and microscopic examination of stomach contents and bee parts was conducted in addition to the fenotypical determination of the origin of the pollen in the body hair and corbicular loads.

Chemical analyses of samples of the poisoning incidents were conducted at the Institute for Ecological Chemistry, Plant Analysis and Stored Products Protection, Julius Kühn-Institut.

Screening and identification of about 200 different active substances in plant and bee samples were generally conducted with GC/MS [Trace DSQ II (Thermo Scientific)] and LC/MS/MS [Triple quadrupole mass spectrometer API 4000 QTRAP (Applied Biosystems MDS Sciex) coupled to a Shimadzu HPLC-system], covering pesticides either authorized in Germany or in other countries of the E. U.

LC/MS/MS was used for the determination of clothianidin and methiocarb in the samples. For quantification the use of matrix-matched calibration with internal standard was necessary. The method was validated by conducting recovery experiments with bee and plant material. The mean recovery at the fortification level of 0.05 mg/kg was 120 % with a relative standard deviation of 2 %. The limit of detection was 0.5 µg/kg for clothianidin in bee matrices and 0.3 µg/kg in plant matrices and 1.0 µg/kg for methiocarb in bee and plant matrices. Further details about the method will be published soon.

Dust in the seed bags, abrasion of seeds

After the causal connection of the bee poisonings with sowing of maize became clear, seed lots were bought from the market and the amount of dust in the seed bags analysed. Originally sealed batches of maize seeds of different varieties and different insecticidal seed treatments were emptied carefully and sieved over a 6 mm sieve and the amount of dust of the whole bags documented. The size of dust particles was separated into the fraction of finer dust <0,5mm and coarse dust. Broken seeds were removed and not weighed.

Residues of the dust were analysed separately for fine and coarse dust particles using liquid chromatography-mass spectrometry with elektrospray ionization (ESI). The LC-MS system consisted of a high-performance liquid chromatograph Perkin Elmer Series 200 and an API 2000 (Applied Biosystems MDS Sciex) with triple quadrupole mass spectrometric detection. Final determination is by LC. The analytes were separated with mobile phase gradient on Synergi Max RP 4 µm 75 mmx4.6 mm i.d. (Phenomenex) using an internal standardisation. The dust samples were extracted with acetone and diluted with internal standard. The limit of quantification for this special screening has been set at 0.1 mg kg-1.

Results and Discussion

Reports and observations of poisoning incidents

A varying extent of damage was observed at different apiaries. The on-site inspection of damaged colonies revealed that 25.3% of 12.174 damaged colonies from 736 beekeepers suffered damages less than 33%. Of the colonies 57.1% had a damage between 34% and 66%, 17.6 % were more than 66% damaged (MLR/LTZ, 2008). In Bavaria, approximately 460 colonies from 36 beekeepers reported bee poisonings in areas with *Diabrotica* eradication programme. The extent of damage to colonies and the level of contamination of an apiary was different due to the individual situation in the surrounding of the colonies; influenced for instance by the distance of maize sowing to flowering plants, the seed batch quality, wind conditions, the type of sowing machine and air outlet, and level of contamination of nectar and pollen, the attractiveness and foraging intensity on highly contaminated crops and the use of uncontaminated crops. During full bee flight in the first days after the beginning of poisonings, many dying and dead bees were found in front of the hives. Returning nectar foragers with symptoms of intoxication were noticed in front of the hives and while entering hives, passing on the contaminated nectar to other bees. During flowering of the contaminated crops, up to thousands of dead and dying bees were discovered daily during bee flight activity. In addition to these acute toxic effects colonies suffered further retarded effects after the end of spring flowering. Some beekeepers did not follow the official recommendation to remove all combs containing pollen from affected colonies. In these cases damages continuing up to several weeks after sowing of maize demonstrated that the contaminated pollen was stored as bee bread, subsequently causing the death of those bees consuming higher amounts of contaminated pollen, like nurse bees. Especially in the early morning dead and dying bees were found in front of these hives. Symptoms of damaged brood were observed, possibly due to poisoning by contaminated food, but also brood damages by the loss of adult and hive bees were observed as brood areas could not be maintained and undercooling of brood areas occurred. In general, colonies recovered after flowering of the contaminated crops had ended; before flowering of maize started, bee keepers raised concerns that residues systemically translocated into pollen of maize could lead to further intoxifications or poisonings.

Bees and bee matrices: The first visual inspection of the samples showed a rather small portion of pollen foragers in many of the bee samples. Analyses for bee diseases revealed that the incidents were not linked with bee diseases or parasites. The microscopic analysis of spores of *Nosema* spp. in 24 samples detected no spores, in 43 samples low amounts of spores (<5), in 15 samples a moderate number (10-30 spores) and in 3 bee samples a high number of more than 30 spores . The Analysis of *Nosema* spores indicated that both bees with and without *Nosema* were equally affected by the poisonings. Furthermore analyses of samples at the CVUA, Freiburg confirmed that the bee samples were not infected with viral diseases or other bee diseases. (Ritter, 2008, pers. comm.). A biotest of contact toxicity with bee samples and larvae of *Aedes aegypti* (n=70) was positive in 91.4%. In some of the bee samples, small coloured dust particles were discovered in the midgut. The fenotypical determination of the origin of the pollen in the body hair and corbicular loads demonstrated that the poisoned bees had foraged on a wide range of plant species and not only on one main crop.

Table 1 Poisoned bees analysed for residues

Range	Bees with clothianidin	%	Bees with methiocarb	%
n =	77	100,0	77	100,0
no residues	6	7,8	52	67,5
0-1 µg/kg	1	1,3	0	0,0
1-5 µg/kg	2	2,6	18	23,4
5-10 µg/kg	25	32,5	6	7,8
10-15 µg/kg	24	31,2	0	0,0
15-20 µg/kg	10	13,0	1	1,3
20-40 µg/kg	7	9,1	0	0,0
> 40 µg/kg	2	2,6	0	0,0
Total with residues	71	92,2	25	32,5
Maximum µg/kg	212,2		18,5	

Chemical analyses of poisoned bees collected by the bee keepers revealed the presence of clothianidin in most bee samples, in fresh pollen collected with pollen traps and in bee bread of damaged colonies sent from the affected regions. The results of the analyses indicated that eight additional samples originating from regions where Poncho Pro had been used were clearly linked to spray applications with other insecticides.

The bee poisoning incidents were clearly linked with the abrasion of an insecticidal seed treatment during sowing of maize. Multiple background residues of further insecticides, fungicides and herbicides were also found in all bee and bee bread samples. In some of the bee and bee bread samples, also methiocarb was found indicating the origin of these active substances from treated seeds. Some of the maize seed lots treated with the high rate of clothianidin were additionally treated with methiocarb to repel birds. Methiocarb deriving from seed treatments was never linked with bee poisoning incidents before 2008 though being used for more than 20 years. The maximum rate of methiocarb treatment to seeds is 150 g a.s./ha. In regions with poisonings the rate of clothianidin was 125 g a.s./ha. In 6 bee samples no clothianidin was detected. In 2 of these bee samples which were sent several weeks after the initial damage, the storage conditions of the samples were not described, and clothianidin was not detected in bees, but in bee bread. In one sample, it was not possible to conclude or exclude a clear link with sowing as only the plant sample had a very low contamination with clothianidin, fipronil and a higher contamination with methiocarb but the bee sample was not contaminated. In another three samples no clothianidin but fipronil was found, possibly by use of imported seeds treated with fipronil.

Table 2 Residues of clothianidin and methiocarb in bee bread samples sent by beekeepers

Range	Samples with clothianidin	%	Samples with methiocarb	%
n =	20	100	20	100
no residues	9	45	2	10
0-1 µg/kg	3	15	1	5
1-5 µg/kg	6	30	1	5
5-10 µg/kg	1	5	7	35
10-15 µg/kg	1	5	5	25
15-20 µg/kg	0	0	1	5
20-40 µg/kg	0	0	2	10
> 40 µg/kg	0	0	1	5
Total with residues	11	55	18	90
Maximum µg/kg	15,5		83,4	

Analyses of 67 bee samples at the LTZ revealed 28.4% without detectable residues, 25.4% of the samples had 1-5 µg/kg, 38.8% had 5-10 µg/kg, 7.5% had 10-15 µg/kg. No higher rates were detected. Some of the samples which had no residues were taken after the end of flowering, as damages were already decreasing or had stopped (Trenkle, 2009, pers.comm.). No detectable residues were found in analysis of 58 of 65 honey samples. In 7 honey samples, only low residues between 1.1 – 2.3 µg/kg were detected (MLR, 2008).

Clothiandin and methiocarb were also found in pollen and bee bread. In 3 pollen samples collected with pollen traps 12.5, 5.4 and 26.4 µg clothianidin /kg, and 27.9, 6.5 and 26.9 µg/kg methiocarb were detected. 8 samples of bee bread had no residues of clothianidin and were not linked with sowing of maize. Clothianidin was not detected in one sample of bee bread, but in dead bees from the same colony. The presence of residues in pollen and bee bread confirms that pollen foragers successfully foraged and stored highly contaminated pollen in the combs. Pollen foragers were most likely less affected by acute poisoning during foraging activity compared to nectar foragers.

Analyses of 117 samples of LTZ, LUFA Speyer and Bayer CropScience (MLR/LTZ, 2008) revealed no residues in 65.8% of the bee bread samples, 6.0% had 1-5 µg/kg, 5.1% had 5-10 µg/kg, 6.8% had 10-15 µg/kg, 0.9% 15-20 µg/kg, 11% 20 -50 µg/kg and 4.3 % had more than 50 µg/kg clothianidin. The maximum was 77 µg/kg clothianidin.

Due to the individual foraging behaviour, the pollen supply of the colonies usually consisted both of uncontaminated pollen and pollen contaminated with varying amounts of residues. It is most likely that especially crops directly neighbouring maize fields were strongly contaminated. Nectar and pollen collected from these field edges were highly contaminated and varying amounts of residues were present in food sources in the surroundings. Several pollen loads are used to fill one cell. It is most likely that individual pollen layers in a bee bread cell show a variation of the residue content, and also a variation between cells. As for a representative residue analysis of a bee bread sample several cells are needed, a partial contamination can not be detected. The analysis may therefore underestimate relevant residues in smaller bee bread fractions. As the pollen may be stored and consumed at a later date, retarded poisonings of bees can occur. This explains beekeepers reports that after rainy periods or periods of low pollen income increased but moderate numbers of bees were observed showing symptoms of poisoning or behavioural abnormalities. In general, after that flowering of directly contaminated plants had ended and other nectar and pollen became available colonies were able to recover well from the damages.

During flowering of maize, beekeepers were concerned that systemically translocated residues might cause new poisoning incidents, but only very few claims of poisoning incidents were reported from bee keepers in the region after sowing of maize. Some beekeepers claiming poisoning incidents suspected these damages were caused by residues of clothianidin in maize pollen. Residue analyses concluded no link with maize but poisonings by spray applications linked with different crops instead. The level of residues present in pollen of maize from plants and in pollen from pollen traps was in the expected range and covered by earlier risk assessment studies, indicating no risk for bees. In a monitoring programme of Bayer CropScience, 250 maize pollen samples had a mean of 3.4 µg/kg clothianidin, in 118 samples of pollen from pollen traps at the monitoring hives a mean of 1.1 µg/kg, in 36 bee bread samples a mean of 1.0 µg/kg was detected (Nikolakis et al., 2009). The residue levels present in pollen of maize were clearly lower compared to residues in pollen of flowering plants during sowing of maize. Three samples of fresh pollen from pollen traps had 1.7-3.0 µg/kg clothianidin. No further poisoning symptoms and no disturbance of colony development were observed during and after flowering of maize. Monitoring of damaged bee colonies in the upper Rhine valley did not show any adverse effects on bee health during flowering of maize. No further adverse effects on the colonies regarding overwintering strength, overwintering success and colony strength in spring 2009 were observed (Liebig et al., 2008). A fair overwintering with no unusual overwintering losses 2008/2009 was reported by the bee keepers from the regions affected by the poisonings in 2008.

Plants: Residues of clothianidin in plants that had received no deliberate insecticidal treatments were detected in apple flowers (n=7), dandelion flowers (n=2) and other wild flowers (n=11) and winter oilseed rape (n=15) collected in the proximity of fields. Clothianidin is also used as seed treatment of winter oilseed

rape. Therefore residues of clothianidin were also detected in samples of winter oilseed rape, but only high residues exceeding by far the background levels that may originate from systemic translocation in the plants, can cause the strong poisonous action.

Table 3 Plant samples with residues of clothianidin and methiocarb sent by beekeepers

Range	Plant samples with clothianidin	%	Plant samples with methiocarb	%
n =	35	100,0	35	100,0
no residues	3	8,6	21	60,0
0-1 µg/kg	3	8,6	4	11,4
1-5 µg/kg	14	40,0	3	8,6
5-10 µg/kg	9	25,7	2	5,7
10-15 µg/kg	3	8,6	2	5,7
15-20 µg/kg	0	0,0	1	2,9
20-40 µg/kg	2	5,7	1	2,9
> 40 µg/kg	1	2,9	1	2,9
Total with residues	32	91,4	14	39,4
Maximum µg/kg	47,8		43,0	

Most of the plant samples analysed contained residues of clothianidin and methiocarb. At the time of first plant samplings by the beekeepers the reason for the damages was not clear. Suspecting damages by spray applications, many beekeepers randomly took samples from flowering crops nearby, without knowing the link with sowing. As the documentation of some of the samples was incomplete, a detailed interpretation of the residue data is not possible for all plant samples. Nevertheless it is most likely that these samples underestimate the maximum levels of contamination that were present in the area, as sampling was not targeted to flowering crops neighbouring maize, but even low residues in flowers except winter oilseed rape indicate that drift of dust was the cause for the residues. Some plant samples were excluded from further analyses as no further gain of information could be expected, or the bee samples concluded no link with dust poisoning.

The possibility of emission of dusts containing active substances from dressed seeds during sowing of imidacloprid-treated maize was already shown by Greatti et al. (2003, 2006). Residues detected were in grass samples between 14 µg/kg and 29 µg/kg, in flowers between 22 and 59 µg/kg (Greatti et al. 2006). In another trial with different varieties and seed treatments residues ranging from 22.4 µg/kg to 123.7 µg/kg were found on flowers, on grass residues ranging from 40 µg/kg to 58 µg/kg were found. High residues in plants samples were also confirmed by analyses of the LTZ Augustenberg (MLR/LTZ, 2008). Target-orientated, bee-attractive flowers from plants neighbouring maize fields were sampled, maximum residues of clothianidin found in apple flowers were 98.5 µg/kg, in winter oilseed rape flowers 94.5 µg/kg and 113 µg/kg in dandelion flowers. One drift study carried out by LTZ examining the residues on winter oilseed rape fields after sowing treated maize in adjacent fields demonstrated residue values of about 100 µg/kg on flowers in 1 m distance, reduced to about 70 µg/kg in about 5 m distance.

Dust in the seed bag

After the causal connection of the bee poisonings and the contamination of plants with sowing of maize became clear, seed lots were bought from the market and the amount of dust in the seed bags analysed.

A high variation was detected for the amount of dust in 82 different seed lots ranging from 2 to about 60 gram per 80.000 kernels (amount for 1 ha) with an average of 3.6 g fine dust and 4.9 g coarse dust. The coarse dust contained mainly larger plant particles (glumes) from seeds of maize which were treated together with the seeds but broken from the seeds. Fine dust mainly seems to appear if the quality of the coating process is not sufficient. The occurrence of glumes depends on cultivars, harvesting time and technique, and intensity of cleaning of seeds before the coating process. Often the glumes were broken in smaller particles

falling into the fine dust fraction. High amounts of dust were also detected in several batches of seed bags of maize by the LTZ Augustenberg (MLR/LTZ, 2008).

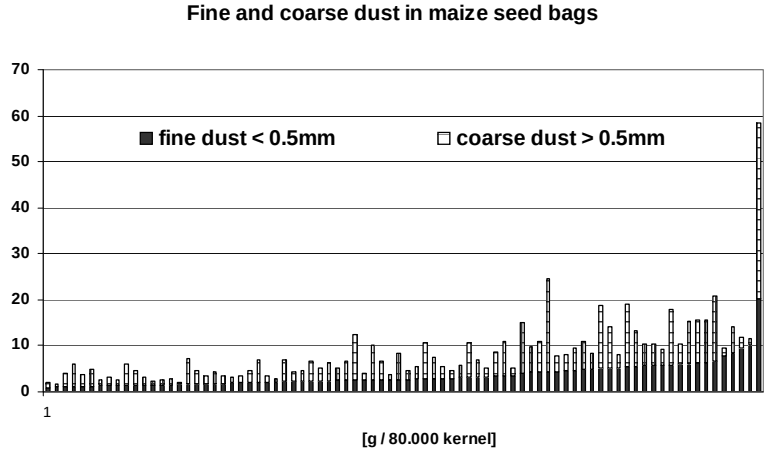

Figure 1 Fine dust <0.5 mm and coarse dust > 0.5mm after slightly sieving of whole seed bags with 6 mm mesh size (different batches, collected from the German maize seed market)

The dust of those seed batches treated with clothianidin (often also with other fungicides and methiocarb) was analysed for clothianidin residues. High residue contents were detected in the dust with significant higher rates in the fine dust compared to the coarse dust (Tab. 4). Lower residues in coarse dust can be explained by more plant material in this dust fraction which dilutes the percentage of clothianidin. Dust treated with the higher rate of clothianidin had significant higher residues. But clothianidin residues in fine dust were not significantly affected by the amount of fine dust per seed bag detected. There is a tendency that higher rates of coarse dust in seed bags of maize reduce the residue content which might be explained by the higher amount of plant material which partly also falls into the fine dust fraction and which dilutes the residue content. Coarse dust containing larger amounts of plant material is less contaminated with clothianidin.

Table 4 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.

		dust < 0.5mm clothianidin		**dust > 0.5mm clothianidin**	
clothianidin / kernel		in %	sd	in %	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)

First analyses in 2008 showed that seeds from different crops vary in the amount of dust. Whereas maize seed batches generally contained dust particles of varying but high amounts, only very low amounts of dust were found in crops like sugar beet or winter oilseed rape. (Heimbach, unpublished). Size and structure of different seeds allow different seed treating techniques and a coating enclosing the whole seed.

The specific sowing technologies for different crops may result in varying amounts of dust blown out, depending on technical solutions by the manufacturers of sowing machines.

Conclusion

The high number of poisoning incidents in Baden-Württemberg and Bavaria in spring 2008 could clearly be linked to the sowing of maize and the abrasion and emission of dust containing the active substance clothianidin. Emission and drift of considerable amounts of dust particles may pose a risk for honey bees when bee toxic substances are used for seed treatments.

Clothianidin was detected in the chemical analyses of poisoned bees collected by the bee keepers but also in samples of plants, fresh pollen and bee bread after the high rate of clothianidin of 125 mg a.s./ha had been used. Some maize seed bags with exceptionally high amounts of dust were found. Dust containing active substance was emitted with pneumatic sowing machines resulting in drift of dust and contamination of bee attractive plants at the time of main nectar flow.

To enable a safe sowing of seed treated crops in the future, the improvement of seed quality and a reduction of emission due to actions taken by seed breeding companies, seed treating companies, corn drilling machinery industry and chemical industry is necessary. The technique of the seed treatment varies for different crops, also the structure of seeds is different. This may cause differences in the amounts of dust. First results show that seeds from different crops vary in the amounts of dust in the seed bag. Technical solutions for avoiding the formation of loose dust must be established to remove all unwanted dust particles before, during and after the coating process. The glume particles originating form the seeds of maize need to be removed as far as possible before the seeds are treated. Proper coating systems and appropriate stickers must be used to ensure that only a minimum of dust can be abraded during handling and sowing of maize seeds. Furthermore, the coating process must guarantee that during handling, transport, and sowing no new dust should be generated. A quality check of treated maize seeds needs to be established after the coating process. The Heubach Dustmeter is in use for 2009 and seems to be suitable for this purpose. Maximum Heubach values need to be defined which ensure a minimum of dust during sowing.

Concerning the future of insecticidal seed treatments, appropriate risk mitigation measures for potential abrasion and dust generation need to be established for crops with a potential of considerable amounts of dust. In addition to an improved seed quality the emission needs to be stopped by technical solutions for sowing machines as far as possible, regardless of the substance. Promising appropriate first technical solutions for drift reduction have been developed, are inexpensive and have proven to be effective to reduce drift for most maize sowing machines. Sowing machines used today are mostly precision pneumatic planters with vacuum singling. In case of maize, especially pneumatic single-seed machines seem to have a high emission potential. The emission of these dust particles with pneumatic single-seed drilling technology needs to be reduced. Most of those machine types centrally blow the air of all rows upward or sideward resulting in a contamination of neighbouring area and therefore are vulnerable to wind drift. Changing the direction of the air from the outlets of the seeders and reducing wind speed seems to be a promising technique to reduce drift. A method was established in the JKI to measure drift reduction due to different modifications of the air exhaust. In autumn 2008 the sowing machines of all well known manufacturers were tested by the Institute for Application Techniques in Plant Protection. For several types of seeders modifications of the air exhaust now directed to the soil were constructed by the manufacturers. The modified setups were tested; those which proved a drift reduction of at least 90 % were registered in the JKI-list “drift reducing maize sowing machines” (Rautmann et al., 2009).

Such drift reduction setup change is compulsory for sowing insecticidal seed treated maize in Germany in 2009.

Furthermore, within a risk assessment the possible amount and drift of dust, and the potential hazard of dust for honeybees should be taken into account. Hazard quotient (HQ) values are in use to describe the risk for honeybees caused by spray applications. The HQ approach or TER- calculations may possibly be used for risk assessment but must be adapted from spray applications to the risk of dust emission, as the allocation, dispersion and deposition of dusts may be different from sprays. Only a fraction of the total active substance is blown out during sowing with dust, but the higher portion of this dust is presumably depositing within a short distance, within few meters of the sowing machine. For the risk assessment, trials on dust emission were formerly conducted but these did not indicate a considerable risk for bees. Only clean seed batches seem to have been used for these trials. To estimate possible adverse effects to honey bees, the development of appropriate new approaches of study designs are required to cover sowing scenarios and generate basic data necessary for improving the effectiveness of the established risk assessment schemes.

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Bee poisoning caused by insecticidal seed treatment of maize in Germany in 2008

Rolf Forster

Federal Office of Consumer Protection and Food Safety (BVL), Department 2 Plant Protection Products, Messeweg 11/12, 38104 Braunschweig
Fon: +49 531 299 3610, Fax: +49 531 299 3005, E-mail: rolf.forster@bvl.bund.de

Abstract

Background: In late April and early May 2008 a bee mortality occurred in parts of South-West Germany, which affected approximately 12,000 colonies of bees, some of them substantially. Immediately after this became known, an intensive search for the causes of these incidences was started.

Results: Very soon, maize seeds which had been treated with the insecticidal substance clothianidin were suspected as a possible cause. Only two weeks later a clothianidin poisoning was confirmed by the JKI. On May 15, 2008 the BVL-authority ordered suspension of the authorisation of a number of insecticidal seed

treatment products, especially those containing neonicotinoid substances such as clothianidin, imidacloprid and thiamethoxam and in addition methiocarb.

Conclusions: For all future authorizations of pesticides used as seed treatments additional conditions for use will be applied for precautionary reasons. These will cover: the use of additional stickers, maximum permissible values for abrasion, where applicable, the prohibition of sowing of treated seeds at wind speed higher than 5 m/s, the obligation to incorporate treated seeds including dusts into or directly onto the soil, the ban of vacuum systems, unless the exhaust air pipe allows for an incorporation of dusts into the soil or directly onto the soil, where applicable.

Keywords: bee poisoning, seed treatment, maize, neonicotinoids

Introduction

In 2007 the western corn rootworm (*Diabrotica virgifera virgifera*), which was classified as quarantine pest by the European Union (EU) in 2003, was found at different locations in Southern Germany, namely Bavaria and Baden-Württemberg. It was therefore essential, in order to eradicate this pest, to control the larvae of the western corn rootworm by using maize seeds treated with appropriate pesticides in 2008. In late April and early May 2008 severe poisonings of honey bees (*Apis mellifera*) were reported in parts of South-West Germany, which affected approximately 12,000 colonies of bees, some of them substantially.

Immediately after this became known, an intensive search for the causes of these incidences was started. For this purpose the Ministerium für Ernährung und Ländlichen Raum (Ministry for Food and Rural Areas) of the federal state of Baden-Württemberg and the local authorities collaborated with the bee-keepers, the laboratory for the investigation of bee incidents at the Julius Kühn-Institute (JKI), the Federal Office of Consumer Protection and Food Safety (BVL) and the plant protection products industry.

Findings

In early May, maize seeds which had been treated with the insecticidal substance clothianidin were suspected as a possible cause and only two weeks later a clothianidin poisoning was confirmed by the JKI.

The regional distribution of the bee damages and the investigation of the seeds also suggested that quality deficiencies occurred in certain lots of maize seeds, which had been treated specifically against the western corn rootworm (*Diabrotica v. v.*).

For this purpose a higher application rate (i.e. 125 g a.s./ha) had been authorised than for the protection against frit-flies and wire-worms.

On a number of expert symposia, organized by the BVL, the details of the honey bee poisonings were presented and it was broadly agreed that the detected clothianidin originated from treated maize seeds where the active substance did not adhere well to the grains.

This minor dressing quality led to a strong abrasion and build up of dust within the seed packages (maximum amounts of approx. 50 g/ha dust containing maximum amounts of approx. 4.5 g a.s./ha clothianidin, Figure 1).

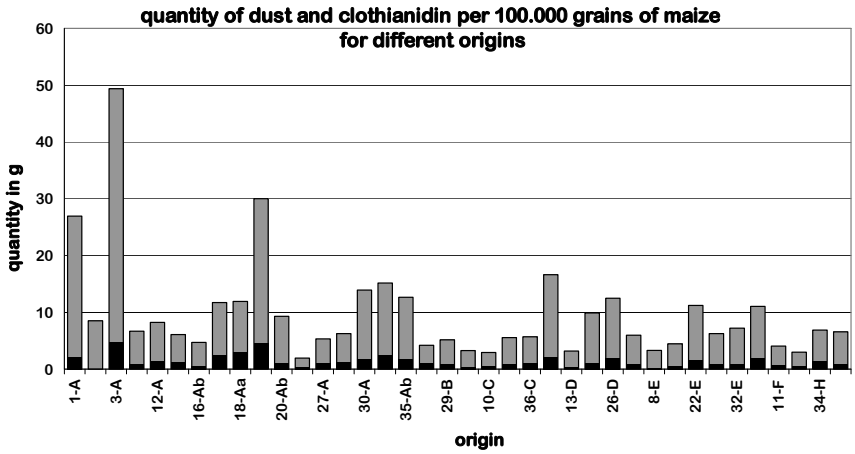

Figure 1 The amount of free dust and proportion of the active substance clothianidin (g per 100.000 grains) contained in 38 seed packages (analyzed by the Landwirtschaftliches Technologiezentrum Augustenberg (LTZ)

In the Upper Rhine Valley pneumatic seeding machines with vacuum systems for single grain application were employed, which, due to their construction, release abrasion dust into the air and onto neighbouring blooming plants, such as oil seed rape an apples (Figure 2).

Figure 2 Emission of contaminated dusts during sowing of maize on adjacent crops (picture by the Landwirtschaftliches Technologiezentrum Augustenberg (LTZ))

The Landwirtschaftliches Technologiezentrum Augustenberg (LTZ) demonstrated that drift of free dusts emitted by pneumatic seeding machines (vacuum systems) onto oil seed rape at 1 m distance amounted up to about 100 µg per kg of oil seed rape (OSR), indicating a severe risk for honey bees. Furthermore the LTZ studied the emission of different types of exhaust air pipes (directed upwards, directed downwards, directed into the soil) and so demonstrated that the emission of dusts into neighbouring fields might be significantly reduced, if the exhaust air pipes were modified so that dusts are incorporated into the soil, e.g. via the fertilizer share (Figure 3).

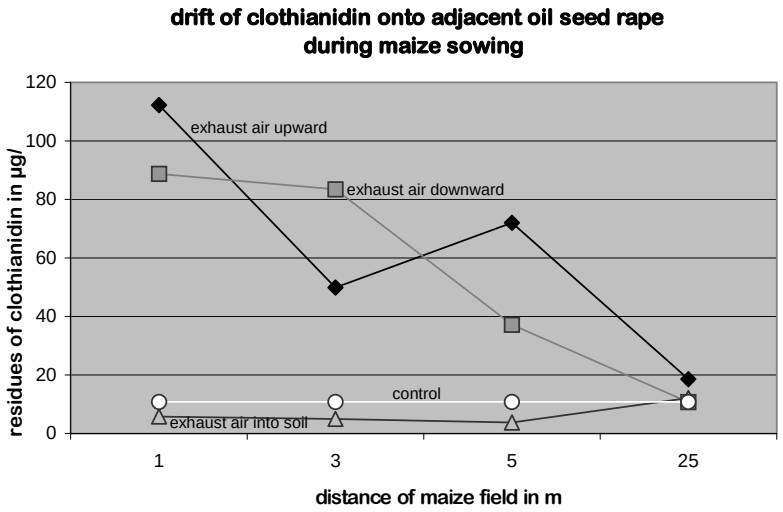

Figure 3 Drift of free dusts (µg per kg) emitted by pneumatic sowing machines (vacuum systems) onto oil seed rape at 1 up to 25 m from the maize field using different types of exhaust air pipes (directed upwards, directed downwards, directed into the soil, control) (analyzed by the Landwirtschaftliches Technologiezentrum Augustenberg (LTZ))

Both of these two main factors coincided with a number of special circumstances which finally generated an acute worst-case-exposure in the South-West of Germany:

- delayed sowings of maize on approximately 15 to 20.000 ha at the same time in the Upper-Rhine valley because of adverse weather conditions,
- followed by dry weather and constant winds which caused a high and directed discharge of dusts into adjacent areas,
- coincidental flowering of oil seed rape, fruits and weeds (e.g. *Taraxacum sp*.), which is considered the most important precondition for exposure of bees to contaminants.

Suspension of the authorisations of insecticidal seed treatment products for maize and oil seed rape

On May 15, 2008, still before the complete clarification of the incidents, the BVL ordered suspension of the authorisation of eight insecticidal seed treatment products:

1. Cruiser 350 FS, BVL-ZA 4914-00 (thiamethoxam)
2. Faibel, BVL-ZA 4704-00 (methiocarb; imidacloprid)
3. Mesurol flüssig, BVL-ZA 3599-00 (methiocarb).
4. Poncho, BVL-ZA 5272-00 (clothianidin);
5. Antarc, BVL-ZA 4674-00 (beta-cyfluthrin; imidacloprid)
6. Elado, BVL-ZA 5849-00 (beta-cyfluthrin; clothianidin)
7. Chinook, BVL-ZA 4672-00 (beta-cyfluthrin; imidacloprid)
8. Cruiser OSR, BVL-ZA 4922-00 (fludioxonil; metalaxyl-m; thiamethoxam)

For precautionary reasons, these measures did not only apply to products for the treatment of maize seeds, but also to products for the protection of rape seed. On May 24, 2008, the Federal Ministry of Food, Agriculture and Consumer Protection banned for a period of 6 months the planting of treated maize by means of certain pneumatic machines for single grain delivery; this ban applied to maize seeds treated with clothianidin or with one of three further insecticides.

In parallel to these immediate measures, the BVL and the JKI intensively dealt with the problem of the abrasion of active substances in seed treatment products. The aim was to clarify which factors play a role in the treatment of seeds and in the sowing process, and how to minimise the damage to the environment. For this purpose the BVL asked authorisation holders for documents and held several expert meetings, during which seed producers, the industry for agricultural machinery, associations and independent experts could express their opinions. The review clearly showed that problems which occurred with maize seeds are not transferable to rape seed in Germany. An experimental study (JKI) on the quantity of dusts in batches of maize and oil seed rape sold on the market demonstrated, that 90 % of the batches for oil seed rape contained less than 1 g/ha of dust whereas for maize 100 % contained more than 1 g/ha dust. More than 99 % of the sowing machines for oil seed rape are either mechanical or pneumatic systems (pressure) where dusts are incorporated into or onto the soil rather than emitted into the air and onto plants.

The risk evaluation by the JKI and the results of the German bee monitoring programme did not produce any evidence for a possible damage to bee colonies due to sowing of oil seed rape. Therefore, on June 25 2008, the BVL reinstated the authorisation for rapeseed (Antarc, BVL-ZA 4674-00, Elado, BVL-ZA 5849-00, Chinook, BVL-ZA 4672-00, Cruiser OSR, BVL-ZA 4922-00). For precautionary reasons additional conditions for use were ordered, such as the use of an additional sticker, in order to minimise free dusts and dusts from abrasion, as well as further labels, such as the prohibition of sowing treated seeds at wind speed higher than 5 m/s, the obligation to incorporate treated seeds including containing dusts or dusts generated during the sowing process into or directly onto the soil, the ban of pneumatic systems (vacuum systems), unless the exhaust air pipe allows for an incorporation of dusts into the soil or directly onto the soil.

For pesticides containing neonicotinoids used for seed treatment of maize, the requirements for an authorization in accordance with Directive 91/414/EEC are currently not fulfilled. Therefore the authorizations are suspended as long as the relevant conditions do not allow for a safe use. Further requirements are:

The chemical companies need to submit new data and risk assessments covering:

- the dispersal of contaminated dusts, including wind erosion,
- new exposure scenarios for contaminated dusts, including toxicity data for dusts,
- abrasion of upgraded formulations, e.g. using stickers, and improved sowing machinery,
- prescriptive limits for free dusts and abrasion, e.g. according to the Heubach-test.

The plant breeding companies need to establish:

- new procedures for seed treatment, especially aspiration of free dusts and use of optimal stickers,
- quality assurance with respect to free dusts and abrasion, e.g. according to the Heubach-test.

The producers of sowing machines need to reconstruct their machines in order to avoid the emission of contaminated dusts.

Future perspectives for the authorisations of insecticidal seed treatment products

The aim of BVL is to close the source of emission by reducing the dusts in the seed bags by 90 % and reducing the emission of remaining dusts by sowing machines by 90 %, in total accounting to a 99 % reduction of emission as far as maize seeds are concerned. The BVL is about to impose a limit for the quality of maize seeds with respect to the abrasion of dusts. For 2009 the maximum permissible value will be down to 0.75 g per 100.000 grains. In addition to that approach of the BVL the JKI is about to establish a list of

'acceptable' sowing machines, reducing dusts in adjacent fields by 90 %, which will be addressed by the respective authorizations.

However, for the authorization of pesticides for seed treatments new data requirements will be defined on a crop by crop basis to take this path of exposure into due consideration:

- data on free dusts and dusts from abrasion for each crop,
- data on sowing machines used and potential emissions.

For all future authorizations of pesticides used as seed treatments additional conditions for use will be applied for precautionary reasons. These will cover:

- the use of additional stickers, in order to minimise free dusts and dusts from abrasion,
- maximum permissible values for abrasion, where applicable,
- the prohibition of sowing treated seeds at wind speed higher than 5 m/s,
- the obligation to incorporate treated seeds including containing dusts or dusts generated during the sowing process into or directly onto the soil,
- the ban of pneumatic systems (vacuum systems), unless the exhaust air pipe allows for an incorporation of dusts into the soil or directly onto the soil, where applicable.

Acknowledgements

I would like to thank all those colleagues who supported the BVL in the research for the causes and the scientific background of these serious honey bee poisonings.for their contribution to further improve the protection of honey bees in the field.

Risks to bees from dusts emitted at sowing of coated seeds: concerns, risk assessment and risk management

Anne Alix, C. Vergnet, T. Mercier

AFSSA-Direction du Végétal et de l'Environnement, 10 rue Pierre Curie, 94704 Maisons Alfort Cedex, France

Abstract

The use of Plant Protection Products (PPP) through seed coating may lead to honey bee exposure mainly in the case of systemic properties, through residues that thus may reach green and flowering parts of growing plants. Incidents occurred in France, Germany and Slovenia. These revealed mortality events in honey bee colonies occurring immediately after sowing of coated seeds which could not be explained by systemic properties. These incidents were related to a loss of active substance from the outflow air fan of pneumatic sowing machines and possible pollution of vegetation in nearby fields.

Investigations were undertaken in France in order to identify the factors responsible of these incidents[1]. A low coating quality was demonstrated, which lead to the emission of higher level of dusts compared to usual coating. Higher levels of residues could also be observed in the dusts generated by the low quality coating compared to a normal one. Further research was performed in Italy, on outflow air from pneumatic seed drills[2], which demonstrated a pollution of plants in the vicinity of sowed areas, at levels directly dependant on the length of sowing duration. This observation leads to recommend a quality control of the dust level at the seed treatment plant.

Specific equipments exists, which may reduce the risks by limiting dust emission during sowing operations. Outflow fans may for example be oriented towards the soil so that dust drift is limited. In addition, deflecting devices may redirect dust to the soil and avoid turbulence and further drift. An efficacy assessment of these devices compared to 'conventional' equipment may be a preliminary requirement to their generalized implementation on seed drills.

Prior to an adaptation of sowing material, the question of the risks posed by sowing dusts to honey bees remains. In France, a dedicated risk assessment has recently been performed for two PPPs to be used as seed coating[3]. Exposure of bees was assessed from dedicated experimental data on dust emission from the coated seeds according to high quality standard. The amount of active substance emitted was determined and used as an application rate estimate in a hazard quatient calculation, further compared to Directive 91/414/EEC trigger and by comparing the drift dose rate on dusts to acceptable exposure levels in tunnel testing. Due to the nature of the risks related to a sowing event, contact toxicity value was preferred. This risk assessment lead to conclude to acceptable acute risks for the products evaluated. Nevertheless, such an assessment may probably be improved and remains a precondition to routinely implemented controls of coating quality, through e.g. dust emission/abrasion tests.

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[1] Commission d'étude de la toxicité, des produits antiparasitaires et supports de culture, procès verbal de février 2004 (http://agriculture.gouv.fr/IMG/pdf/avisctweb200401.pdf)
[2] Greatti M., Barbattini R., Stravisi A., Sabatini A. G. and Rossi S., 2006. Presence of the a.i. imidacloprid on vegetation near corn fields sown with Gaucho dressed seeds. Bulletin of insectology 59 (2): 99-103.
[3] AVIS du CES relatif à une demande d'autorisation de mise sur le marché de la préparation Cruiser à base de thiaméthoxam, de la société Syngenta Agro SAS, dans le cadre d'une procédure de reconnaissance mutuelle.
Avis du CES relatif à une demande d'autorisation de mise sur le marché de la préparation Poncho Maïs à base de clothianidine, de la société Bayer Cropscience France, dans le cadre d'une procédure de reconnaissance mutuelle (http\\www.afssa.fr).

An effective risk management approach to prevent bee damage due to the emission of abraded seed treatment particles during sowing of seeds treated with bee toxic insecticides

Alexander Nikolakis, Andrew Chapple, Reinhard Friessleben, Paul Neumann, Thorsten Schad, Richard Schmuck, Heinz-Friedrich Schnier, Hans-Jürgen Schnorbach, Ralf Schöning, Christian Maus*

Bayer CropScience AG, Alfred-Nobel-Str. 50, 40789 Monheim, Germany
*Tel.: +49 2173 38 3750; Fax: +49 2173 38 4981. E-mail: christian.maus@bayercropscience.com

Abstract

In spring of 2008, a bee incident occurred in the Upper Rhine Valley (Germany) during drilling of corn: bees were exposed to dust from abraded particles of the seed-coating containing the insecticide clothianidin. An inspection of drilled seed batches for resistance to abrasion and a geographical correlation analysis between specified seed batches and reported bee damages revealed that the incident was caused by improperly dressed batches of corn seeds with excessive abrasion of seed treatment particles which were subsequently emitted via the outlet air stream of the pneumatic drilling machines. Concerns raised by local beekeepers regarding effects on bees from foraging in seed-treated corn fields during bloom could be dispelled by a large-scale survey of clothianidin residues in pollen from the treated crop and an accompanying monitoring of bee hives exposed to flowering corn fields. In order to ensure the bee safety of seed-dressing products, technical improvements of seed treatment quality and drilling technology were developed resulting in a minimization of formation and emission of dust from abraded seed treatment particles. The efficacy of these improvements was proven in field trials.

Keywords: seed treatment, drilling machines, corn, clothianidin, dust, honey bees

Introduction

In late April and early May of 2008, numerous cases of increased bee mortalities were recorded in the Upper Rhine Valley (SW Germany). Typically, the affected bees showed symptoms of acute intoxication, in most cases these effects were seen in adult bees only. Approximately 11,500 bee hives were affected. The investigation of the incident was started by regional and Federal authorities immediately after the first records of conspicuous mortality. From the beginning, there were indications which linked the increased

mortalities with the drilling of corn, which took place simultaneously in the affected region. In dead honeybees and samples of vegetation adjacent to drilled corn fields, residues of clothianidin were detected. Clothianidin is a neonicotinoid insecticide contained in the seed-dressing product Poncho Pro® (Clothianidin FS 600, 1.25 mg a.s./kernel), which is applied as a seed-dressing product to corn seeds and was used in the Upper Rhine Valley for control of the western corn rootworm (*Diabrotica virgifera*), an economically devastating pest in corn. Some farmers in the affected area reported unusually high amounts of dust in the bags of treated corn seeds and the emission of red dust during the drilling of these seeds. These reports provided indications that dust from abraded particles of the seed-dressing, which contained the intrinsically bee-toxic clothianidin, was released during the drilling process with the outlet air of pneumatic drilling machines and deposited on flowering, bee-attractive crops and weeds in adjacent vegetation strips and fields where bees were exposed during foraging.

A coincidence of several worst-case factors aggravated the impact of this excessive dust emission: the patchy landscape structure of the Upper Rhine Valley where many small-sized corn fields are located in a diverse agricultural landscape with canola fields, orchards, and other bee-attractive crops, the unusual climatic conditions in the year 2008 due to which the corn drilling and the flowering season of some crops like canola, several orchard crops, and others took place simultaneously, and dry, windy weather during the drilling season, which enhanced formation and drift of dusts.

This paper presents in its first part the results of a detailed analysis of the incident. A basic understanding of the factors causing this incident was seen as the key prerequisite to identify appropriate measures to reliably prevent a repeat of such accidents. This part likewise addresses potential risks posed by the systemic nature of the insecticidal component of the seed treatment product Poncho Pro® to honeybees. In response to massive concerns raised by the local beekeeper community, a residue survey was performed on corn pollen which was accompanied by a monitoring exercise of bee colonies which were installed on three locations within the Upper Rhine valley and regularly inspected. In the second part, this paper summarizes the outcome of a joint research initiative of seed-breeding companies, the drilling machinery industry and the agrochemical industry aiming at the development of appropriate technical solutions to ensure safety of seed-dressing products for honeybees and wildlife. Finally, the results of field trials conducted in order to evaluate the effectiveness of the developed optimizations are presented, and an exemplary bee risk assessment under consideration of the described mitigation measures is outlined.

Results and discussion

Investigation of the Incident

Geographical analysis of correlation: A quantitative analysis was performed with the goal to substantiate or disprove the assumption that an excessive emission of abraded seed treatment particles was the key factor causing the bee incident. In an interview survey, farmers had reported that during the sowing process considerable quantities of dust were generated by the sowing machines, and that this dust had been visibly emitted in the environment with the outlet airstream of the pneumatic drilling machines. Dust subsequently deposited also onto bee forage plants.

Georeferenced data on bee damage and clothianidin residue detects were compared with data on regional sales of Poncho Pro® treated seeds and the seed treatment quality (e.g., resistance to abrasion in standardized laboratory tests), as well as the drilling machineries used. Furthermore, data on land use (e.g., occurrence of corn and canola fields) and land cover (e.g., riparian zones of water bodies) were used, e.g., to characterize the occurrence of bee forage plants. Data were obtained from the Ministry of Food and Rural Land (MLR) and the Regional Councils of Stuttgart and Freiburg (RPS, RPF), and from laboratories of Julius-Kühn-Institut, Braunschweig (JKI), Landwirtschaftliches Technologiezentrum, Augustenberg (LTZ), and Bayer CropScience. Data processing was done in close cooperation with MLR and RPS.

The obtained data set covered the entire Upper Rhine Valley and the Lake Constance region. Information about dust formation during handling and sowing, the Poncho Pro® treated seed varieties (and batches) applied, the types of drilling machines used, as well as the occurrence of bee damage in the relevant area were surveyed by interviews of local farmers.

The analysis of local farmer reports (covering about 2,600 ha in the Upper Rhine Valley and about 600 ha in the Lake Constance region) indicated that in a number of cases poorly treated batches of Poncho Pro® treated corn seeds were sown (here called 'deficient batches') with pneumatic drilling machines resulting in bee damages in the surroundings. In some cases where seed varieties of appropriate seed treatment quality were applied with pneumatic drilling machines no bee damages were recorded, although bee hives and bee forage plants were present in the vicinity of the applied fields. A few cases where “deficient” batches were drilled with machinery of low dust emission potential (here: mechanical drilling machines) did not result in bee damage (presence of bees were confirmed for the respective local areas) indicating that the exposure can be reduced to acceptable limits by an appropriate application technology.

At the scale of the Upper Rhine Valley, a geographical comparison between the density of corn fields drilled with Poncho Pro® treated corn seeds (no differentiation of varieties or seed varieties) and the occurrence of bee damage shows (Figure 1), that in 33 municipalities no bee damage was recorded despite Poncho Pro® treated seeds had been drilled in significant amounts. Since the landscape of the Upper Rhine Valley is characterized by small-scaled and diverse cropping structures and other land uses (e.g., meadows, grassland with fruit trees, wood, etc.) and likewise contains varieties of areas with shrubs vegetation (e.g., along water bodies), it is reasonable to assume that bee forage was generally available. Therefore, it is highly unlikely that bees should not have foraged specifically in these regions since the entire valley is densely populated with bee hives. Likewise, in the Lake Constance region, in only 1 of 35 municipalities where Poncho Pro® treated seeds had been sown, a bee damage was reported. An effect of corn drilling technology could not be analyzed on this scale, as (standard) pneumatic drilling machines were predominately used across the whole region (applies also to the Lake Constance region).

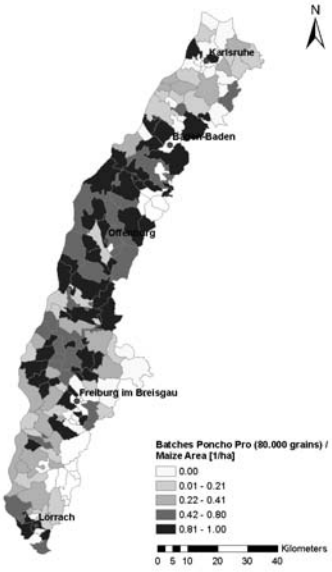


Figure 1 Maps of the Upper Rhine Valley showing the use density of Poncho Pro®-treated corn seeds (left) and the abundance of cases of bee damage (right) on municipal scale.

From this geographical correlation analysis it can be concluded that the recorded bee damages are not related to the use of Poncho Pro® treated corn seeds per se but to the use of "deficient" batches of certain seed varieties which resulted in an excessive emission of abraded seed treatment particles and, by depositing on adjacent bee forage, in critical exposure levels for honeybees.

The relationship between seed treatment quality and the abundance of bee damage was further investigated in a two-step approach: In the first step, regional sales data of specified Poncho Pro®-treated corn seed varieties were correlated with recorded bee damage. This analysis was conducted on municipal level to minimize uncertainty due to bee activity radius and cross-border use of batches. In the second step, abrasion resistance of the analyzed seed batches was determined in standardized laboratory tests (see: Determination of the abrasion resistance of the final seed coating) and related to the correlations obtained in the first step.

In figure 2, the correlation coefficients between the use density of PonchoPro® treated seeds and the abundance of records of bee damage in the same municipality are shown for the top ten seed varieties regarding use densities across the entire Upper Rhine Valley (in decreasing order, i.e. variety 1 has the highest overall use density). These top ten seed varieties cover >75% of the market. The red bars in Figure 2 show the correlation between absolute use densities of the respective seed variety and the abundance of bee damages in the same municipality. A positive correlation indicates that batches of the respective seed variety had received an improper seed treatment. The green bars show the correlation between the market share of a variety and the abundance of bee damages in the same municipality. Negative correlations indicate that the used batches of the respective seed variety had received an adequate seed treatment. Although the simultaneous use of different seed varieties in the same locality and the heterogeneity of a variety regarding the seed treatment quality of different batches substantially increased the data variability, significant correlations could be detected for 9 out of the ten evaluated seed varieties indicating the high relevance of the investigated parameter, i.e. the seed treatment quality.

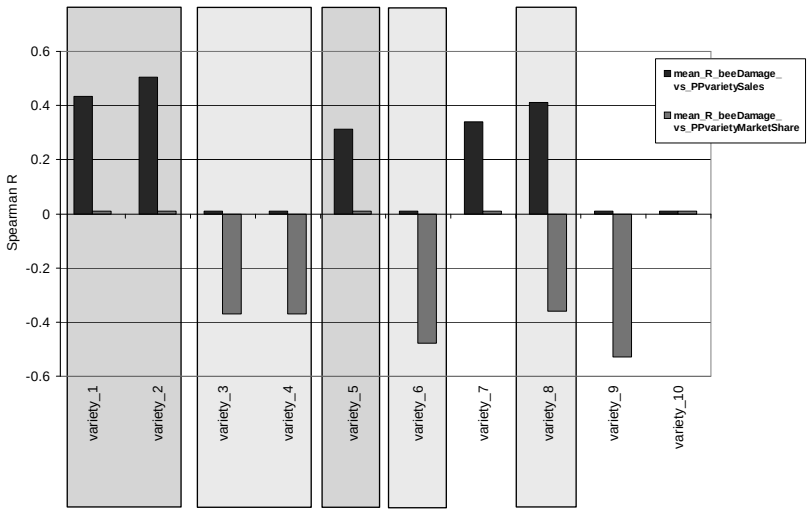


Figure 2 Spearman rank order correlation coefficient R (bars) and results of abrasion resistance tests (rectangles) for batches of corn seed varieties used in the Upper Rhine Valley in 2008. The red bars show the correlation coefficient for the regional use density of a variety and the abundance of bee damage records, the green bars show the correlation coefficient between the market share of a variety and the abundance of bee damage records. The red shaded boxes specify seed variety batches that have shown low resistance against abrasion in standardized laboratory tests, the green shaded boxes indicate tested batches of good seed treatment quality (in agreement with user reports).

The results of the abrasion resistance tests in the laboratory and the user reports on seed treatment quality were highly consistent. The abrasion resistance properties of the investigated batches of several relevant seed varieties are sketched in to figure 2. For seed varieties which correlated positively with the abundance of bee damages, "deficient" batches (red shaded boxes) were identified, whereas varieties which correlate negatively with the abundance of bee damage consistently showed appropriate seed-dressing quality (green shaded boxes). On first glance, seed variety 8 displays contradictory data. However, the use density of this variety strongly correlated with the use density of seed variety 2. Variety 2 contained "deficient" batches and had a significantly higher market share than variety 8. Accordingly, the negative impact of variety 2 explains the positive correlation result for variety 8 regarding use density. The negative correlation between market share and abundance of bee damages for variety 8 (green bars) is consistent with the finding of good abrasion resistance for the investigated batches of this variety.

According to similar data analysis, the single bee incident recorded in the Lake Constance region was most likely also linked with the use of a seed variety with batches of deficient seed treatment quality.

Therefore, the consistent correlations obtained for the relationship between the use density of PonchoPro®-treated seed with "deficient" batches and the abundance of bee damage strongly suggest that an improper seed treatment in combination with the use of the standard pneumatic sowing equipment was the main reason for the bee incident in the Upper Rhine Valley.

Pollen residue survey and hive monitoring exercise - effects of exposure of bee colonies to Ponchopro® treated corn pollen in the upper Rhine valley: Clothianidin is a systemic compound which translocates from the seed surface into the growing plant. Traces of residues may also be found in bee-relevant matrices from treated plants like pollen. In response to the incident during corn drilling, beekeepers raised the concern that bees might encounter systemic residues of the compound via pollen during the flowering period of the corn and that they could thereby be exposed a second time to harmful levels of clothianidin. In order to address these concerns, a large scale monitoring project was conducted in summer 2008. It basically consisted of two parts:

1. Sampling and residue analysis of pollen from treated corn on 50 fields at 5 different locations across the Upper Rhine Valley.
2. Bee health monitoring: on three of the residue sampling sites, bee hives were set up next to treated fields and surveyed for potential effects (conducted by Dr. G. Liebig, University of Hohenheim).

The five locations for pollen sampling were chosen with the focus on where bee incidents had been recorded during the corn drilling season (for distribution of sampling locations see Figure 3). At each of these locations, five corn fields were selected which were grown from PonchoPro®-treated seeds. From each of these fields, five individual corn pollen samples were taken from the crop during flowering period, so in total 250 samples were taken and subsequently analyzed for residues of clothianidin.

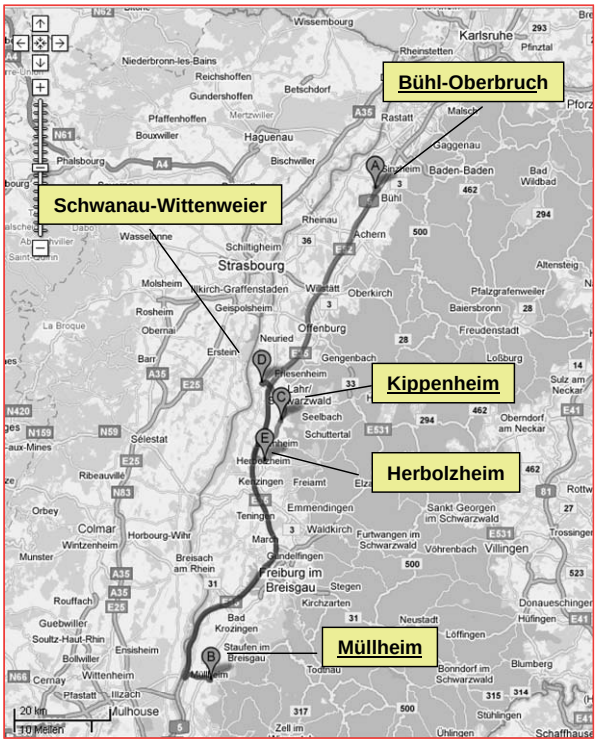


Figure 3 Location of the pollen sampling and the bee hive monitoring sites in the Upper Rhine Valley. Locations with underlined names are both pollen sampling and monitoring sites.

Residue levels in pollen sampled from the crop ranged from < 0.3 µg/kg (Limit of Detection) to 10.4 µg/kg clothianidin, with a mean residue level of 3.4 µg/kg.

For the bee colony monitoring exercise, 15 bee hives of different types, ages and constitutions were set up next to one of the monitoring fields at each of three of the sampling sites (Müllheim, Kippenheimweiler and Oberbruch, see Figure 3) shortly before the corn started to bloom. These colonies were closely monitored for their development and their health condition during the flowering period of corn and until the beginning of the overwintering season. The monitoring colonies developed well and no indications of an adverse effect related to an exposure to harmful chemical residues were found (Liebig et al. 2008). [1]

Along with the hive assessments, samples of hive matrices were taken for residue analysis. In samples of pollen from pollen traps at the monitoring hives, residue levels between < 0.3 µg/kg (LOD) and 11.4 µg/kg clothianidin were found (mean: 1.1 µg/kg, 118 samples). Samples of bee bread from the monitoring hives contained residue levels between < 0.3 µg/kg (LOD) and 3.3 µg/kg (mean: 1.0 µg/kg, 36 samples). The proportion of corn pollen in the pollen traps attached to the hives was very variable between different exposed colonies; on average, the share of corn pollen in the overall collected pollen was 22%, however, some colonies collected virtually no corn pollen, whereas others intensely foraged in the crop. The colony with the highest proportion of corn pollen among the foraged pollen collected 80% corn pollen (LIEBIG et al. 2008). [1]

No residues were detected in samples of dead bees from the monitoring hives with exception of two out of 38 sub-samples which showed a residue level of 1.2 µg/kg.

The residue levels found in pollen were consistent with previous findings in regulatory studies submitted for the national authorization (unpublished data); likewise the absence of any adverse effects in the exposed colonies confirms the conclusion of the regulatory risk assessment, and is consistent with the finding from several previous higher tier studies that exposure of bee colonies to dietary concentrations of clothianidin up to at least 20 µg/kg does not cause any adverse effects (Schmuck & Keppler 2003).[2]

From the pollen residue survey and the bee hive monitoring it can be concluded that systemic residues of clothianidin in corn pollen from Poncho Pro®–treated plants do not pose a risk to bee colonies.

As it was outlined in the previous chapters, the key conclusions that could be derived from the incident analysis were that the bee incident was caused by exposure of bees to abraded seed treatment particles from improperly treated seed batches, and that there is no risk from systemic residues in corn pollen of seed-treated plants. As a consequence of this, effective risk mitigation measures have to focus on two core aspects:

- **Seed treatment quality:** optimization of adhesivity of seed treatment products on treated seeds in order to reduce abrasion.
- **Seed drilling technology:** minimization of emission of abraded seed treatment particles to off-crop habitats.

Development and effectiveness of these mitigation measures are outlined in the following chapters.

Improvements in seed treatment quality and drilling technology

Optimization of seed-dressing qualities: As shown in the analysis of the bee incident in the Upper Rhine Valley, the quality of the seed coating is one of the key factors in avoiding contamination of the environment through abrasion of dust particles containing active ingredients from the seed treatment coat. Seed treatment in general is the process of applying fungicidal and/or insecticidal seat-dressing products onto various types of seeds. Today, the majority of seed treatment products or mixtures are applied as liquid slurry on seeds.

Factors influencing seed coating quality: The main factors influencing the quality of the seed coating in terms of dustiness / abrasion resistance are:

1. the quality of the seeds before the actual seed treatment process,
2. the technical and chemical composition of the used seed treatment formulation,
3. the employed seed treatment machinery and
4. the application recipe

- **Quality of seeds** before the actual seed treatment process: The most important factor is seed cleaning before treatment of seeds. Seed should be free of any organic dust particles as these will greatly affect the dustiness of the treated seeds at a later stage. As any movement of untreated seed will generate dust, an adequate aspiration system is important to remove all dust particles before the seed enters the seed treatment machine.
- **Formulation:** The quality of formulation of the seed treatment products used plays an important role. The main parameters are besides the particle size of solids (i.e. active ingredients, pigments etc.) the content of appropriate polymers (so called “stickers”) in the formulation to enhance the intrinsic adhesiveness.
- **Seed treatment machinery:** Corn seed is commonly treated with modern batch treaters as they offer high flexibility for adjustment and fine tuning of the treatment process according to seed type, seed quality and application recipe used.
- **Application recipe:** Besides the factors mentioned above, the final recipe of the final seed treatment slurry is amongst the most important factors influencing the final quality of the seed coating. Depending on market requirements usually a combination of different seed treatment products (fungicides and insecticides) at varying application rates are applied. Thus, application recipes are often complex and the total amount of products to be applied can vary significantly. In order to ensure a good adhesion of these products on the seed the addition of supplementary and appropriate adhesives (film-coatings) to the final seed treatment slurry is mandatory.

As shown in Figure 4, the addition of adhesives can significantly reduce the abrasion of dust from treated seeds.

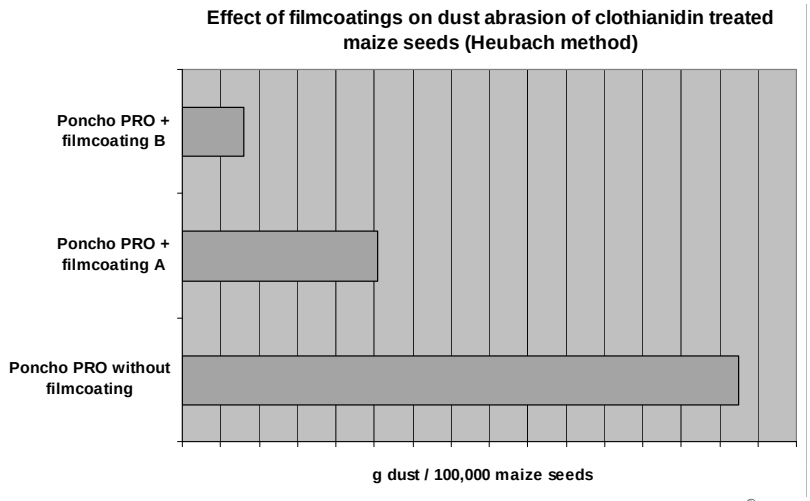


Figure 4 Effect of various film-coating products on the dust abrasion of clothianidin (Poncho Pro®) treated corn seeds

Depending on the seed type, the seed treatment products and their combination, the right adhesive at the optimum application rate has to be chosen to generate treated seeds with a high resistance against abrasion. As the surface properties and the geometry of different seed types (corn, canola, cereals, cotton, sunflower, vegetables, etc.) differ significantly, specific adhesives are designed for each seed type.

Determination of the abrasion resistance of the final seed coating: In order to quantitatively measure the abrasion resistance of treated corn seeds, the Heubach dust abrasion test has been identified as a viable test method which allows best for standardization of dust abrasion measurements within the seed industry, the crop protection industry and independent laboratories, including authorities. Ready-to-use Heubach - dustmeter equipment is commercially available (Figure 5).

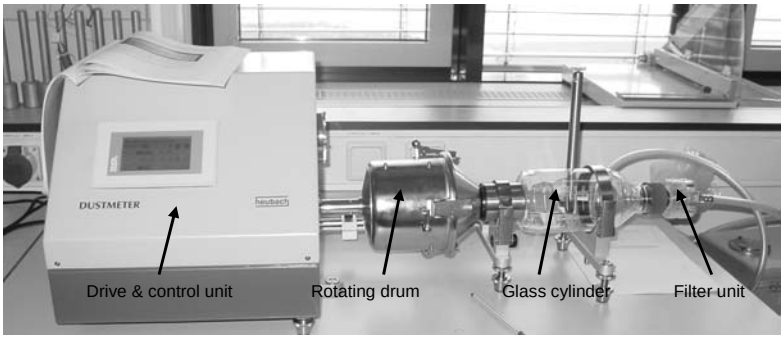


Figure 5 Heubach dustmeter equipment

Moreover, as the Heubach - dustmeter measures gravimetrically the total amount of abraded dust, testing of treated seeds is quick and inexpensive, as no analytical chemistry is involved.

The working principle of the Heubach - dustmeter is that coated seeds are mechanically stressed inside a rotating drum, thus simulating mechanical stress which coated seeds routinely experience in commercial practice, e.g. via bagging, transporting, sowing etc.. A vacuum pump creates an air flow through the rotating drum, glass cylinder and the attached filter unit. Through the airflow, abraded dust particles are finally collected on a filter-disc inside the filter unit (Figure 6). While floating dust particles settle on the filter disc, coarse non-floating particles are separated and collected in the glass cylinder. The amount of floating dust finally collected on the filter disc is the so-called Heubach-value (HV), which is generally expressed - in case of treated corn seeds - as g dust/100,000 seeds (the amount of dust can also be expressed related to the weight of seeds, as g dust/100 kg seeds).

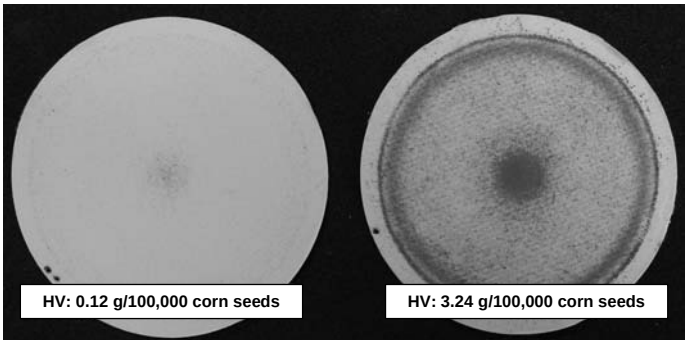


Figure 6 Collected dust deposits on Heubach filter discs

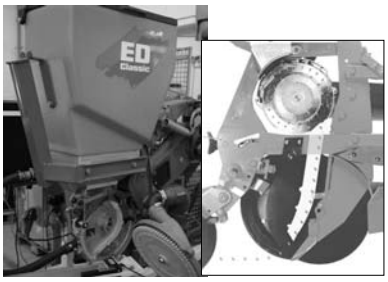
Stewardship measures of bayer cropscience after the bee incident: As an insufficient seed treatment quality has been identified as the main reason for the bee incident in the Upper Rhine Valley in Germany in 2008, Bayer CropScience has initiated an extensive stewardship program for improving seed treatment quality, by

- advising all seed companies which receive insecticidal seed treatment formulations from Bayer CropScience, to implement where necessary, adequate measures to assure maximum cleanliness of the seeds entering the seed treatment process,
- initiating training programs for operators of seed treatment machinery all across Europe, in order to further improve the correct setting of machinery parameters, e.g. mixing time, which may significantly affect the final seed coating quality,
- assisting European seed treatment companies in identifying and choosing adequate adhesives / film-coatings, to achieve a maximum adhesion of the seed treatment products on the seed and to minimize dust abrasion,
- requesting samples of treated seeds from each seed-treatment facility during the start-up period of this stewardship program, before selling insecticidal seed treatment products on commercial scale, in order to verify whether the initiated stewardship measures have been adequately transposed to the actual seed treatment processes
- taking the initiative to provide Heubach - dustmeter equipment to various independent laboratories specialized on seed coating quality investigation in various countries, in order to offer widespread services for Heubach dust measurements to seed treatment companies across Europe (moreover, Bayer CropScience provides Heubach test services at its headquarter and country subsidiaries, where applicable).
- training the laboratory personnel involved in dust measurements to correctly implement Heubach dust abrasion measurements.

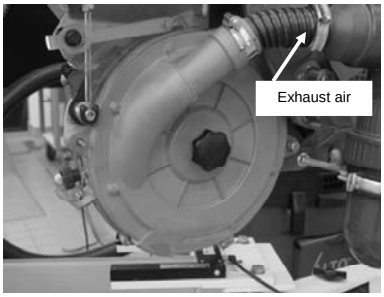
Conclusion on seed treatment quality: Bayer CropScience has initiated extensive stewardship measures all across Europe. The initiative aimed to rise awareness within the seed treatment community to pay particular attention to the abrasion resistance of the final seed coating and to provide expert knowledge and assistance, where required, to assure "Good Seed Treatment Practice".

Modifications of the drilling machinery: Based on the geographical correlation analysis outlined above, insufficient seed treatment quality in combination with standard vacuum-pneumatic sowing equipment has turned out to be the main reason for the bee incident in the Upper Rhine Valley. Moreover, the geographical correlation analysis further revealed that in cases where low-drift technology was used, bee damages have not been reported, even in case inappropriately treated seeds were sown.

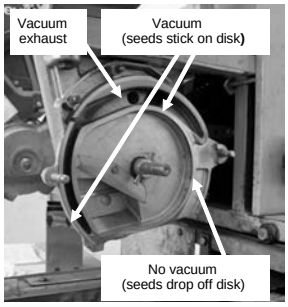
Principle of vacuum-pneumatic corn sowing: Corn is precision-drilled via so called single-kernel sowing devices. To achieve a precise deposition of the seeds in the soil, all vacuum-pneumatic sowing machines (standard and modified) aspirate corn seeds from a deposit via suction pressure, generated by a central fan, on a perforated disk. On the individual perforations of this disk, corn seeds are separated / individualized by sticking to the holes as long as the negative pressure (vacuum) is sustained. Due to the forward movement of the sowing disk, individualised seeds will loose their contact to the vacuum and will therefore finally drop into the furrow, one after the other with a concrete spacing (Figure 7).



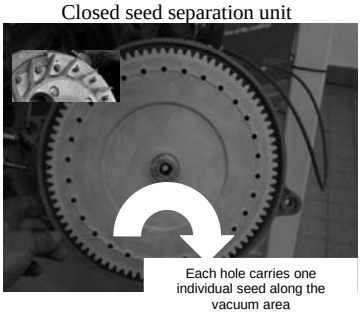
Vacuum-pneumatic sowing device (open seed-separator)



Closed seed separation unit



Vacuum area in separator (without perforated disk)



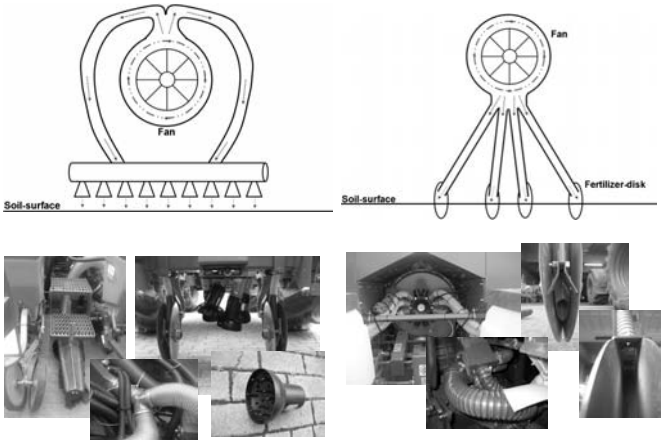
Perforated disk

Figure 7 Principle of pneumatic single-kernel corn sowing

Standard vacuum-pneumatic drilling machines: By spring 2008, standard vacuum-pneumatic sowing devices were state of the art technology for corn sowing in Germany, comprising high market shares. By using this technology, the resulting exhaust air, which contains varying quantities of abraded seed treatment particles (depending on the machinery type and the quality of the corn seed-coating), is emitted with a high flow-off velocity from one single outlet into the air. The air-stream outlet is generally placed directly on the fan, approximately 1.5 - 2 m above the ground. This construction allows for a rather huge dispersion of abraded seed-coating particles in the environment, particularly in the case of an inappropriate seed-coating quality (Figure 8).

Figure 8 Standard vacuum-pneumatic drilling machine with an upward directed air-stream outlet, directly from the fan

Modified vacuum-pneumatic drilling machines: In a co-operative approach, engineers and application specialists of Bayer CropScience and of various manufacturers of vacuum-pneumatic sowing equipment have developed during 2008 concepts of an effective machinery modification, in order to transform existing vacuum-pneumatic drilling machines into low-drift sowing equipment by means of modification kits. Although the developed modification kits differ e.g. in appearance, dimension and exact technical set-up, all modifications follow the same principle approach: the total air-stream generated by the fan to maintain the suction pressure - which was formerly ejected from one single outlet with a high flow-off velocity (see above) - is now divided via several tubes of a rather large cross-sectional area into sub-streams, which are finally released close to the ground. On ground-level, the exhaust air is released via diffusers, cushions or within fertilizer-disks - with or without supporting the fertilizer flow. Overall, the exhaust air is not longer ejected into the environment from approximately 1.5 - 2 m above the ground, but rather gently released close to the soil surface (Figure 9).



Release of exhaust air close to the ground by means of cushions or diffusers

Release of exhaust air close to the ground within fertilizer-disks

Figure 9 Modified vacuum-pneumatic technology

Achievements: Finally, a series of different modified vacuum-pneumatic drilling machines from various manufacturers could be completed right in time to be subject to field testing in summer 2008, in order to investigate whether under field conditions relevant for the commercial corn sowing practice, the developed low-drift technology added to existing vacuum-pneumatic sowing equipment will in fact lead to a significantly reduced off-crop exposure, as intended (for results see: Experimental approach: reality check of the effectiveness of improvements in a drift field trial). Moreover, in addition, independent tests thereafter, the official German Federal authority in charge of approving low-drift technology for both, spray-application and seed-sowing devices (JKI), further examined the five developed modification kits together with other modification kits provided until autumn 2008, for their effectiveness in drift reduction.

Overall, following intensive efforts of the engineers and application specialists of both, machinery manufactures and Bayer CropScience, ≈98% of the European manufactures of vacuum-pneumatic corn sowing machines are now able to provide modification kits for their existing fleet of vacuum-pneumatic sowing equipment. The costs of the modification-kits are generally in the range of several hundred € and the kits can be easily fitted to existing machinery in professional service centres.

Experimental approach: reality check of the effectiveness of improvements in a drift field trial

Study setup and results: In summer 2008, Bayer CropScience conducted an extensive field dust drift study with Poncho Pro®-dressed corn seeds. Overall, more than 70 ha of agricultural land, typical for corn growing under European conditions has been employed for the test program. The machinery under investigation comprised a series of different corn sowing equipments, involving a realistic worst-case unmodified vacuum-pneumatic corn drilling machine as a reference together with five modified vacuum-pneumatic drilling machines of various manufactures. The aim of the modification was to implement low-drift technology to vacuum-pneumatic drilling machines. In addition, also one corn drilling machine which operates with compressed air as well as one mechanical corn sowing machine have been tested. The latter two machines (compressed air and mechanical) were commercially available and not modified, however, supposed to apply low-drift technology due to their specific technical setup. For all investigations, each drilling machinery was tested in the field by sowing dressed corn seeds on an area of approximately 1.0 ha at a drilling rate of 80,000 seeds/ha. In order to investigate and compare the performance of the supposed low-drift drilling technologies in terms of off-crop exposure, the drilling equipment under investigation was uniformly

operated with a seed-coating quality, characterized by a measured dust abrasion value of 1.2 g dust/100,000 corn seeds, as determined by the Heubach dust abrasion test. In the following, this particular seed treatment quality is referred to as "HV 1.2 - seeds" (= seeds with a Heubach Value of ≈1.2 g dust/ 100,000 corn seeds). A better seed-treatment quality than HV 1.2 has not been tested with the low-drift drilling technology due to constraints with the analytical quantification of the emitted dust, particularly at greater distances from the drilling area. Moreover, the influence of the seed-coating quality in terms of off-crop exposure has been investigated with the realistic worst-case unmodified vacuum-pneumatic corn drilling machine (= not low-drift). This machine has been operated with two seed-treatment qualities, i.e. with HV 1.2 - seeds and with HV 0.1 - seeds (= seeds, with a Heubach Value of ≈0.1 g dust/100,000 corn seeds).

At various distances adjacent to the drilling area, Petri-dishes and passive dust-drift collectors were installed in the off-crop sampling area during the drilling procedure. Whereas the Petri-dishes were placed on the soil surface to collect the ground-deposable dust fraction ("primary drift"), the passive dust-drift collectors were installed at various heights above the ground to collect the airborne dust fraction ("atmospheric drift"). After drilling was completed, the samples were collected. Moreover, in order to investigate whether the dust that deposited during sowing within the drilling area will be dislodged from the soil surface and transported downwind ("secondary drift"), a further set of Petri-dishes was installed downwind in the off-crop sampling area after the sampling of the Petri-dishes for the primary drift, to collect dust, potentially dislodged from the soil surface. After an exposure period of 24 hours, these Petri-dishes were collected. The samples were processed in the analytical laboratory of Bayer CropScience AG; the content of clothianidin was determined by using High Performance Liquid Chromatography coupled with tandem mass-spectrometry (HPLC-MS/MS). The results of the study are depicted in Figure 10 - 13.

Discussion of the study results: The comparison of different seed-coating qualities (quality in terms of abrasion resistance as measured by the Heubach abrasion test) on an identical, unmodified, vacuum-pneumatic corn drilling machine revealed that seed-coating quality is a major factor which significantly impacts both, ground deposition and atmospheric drift (Figure 10).

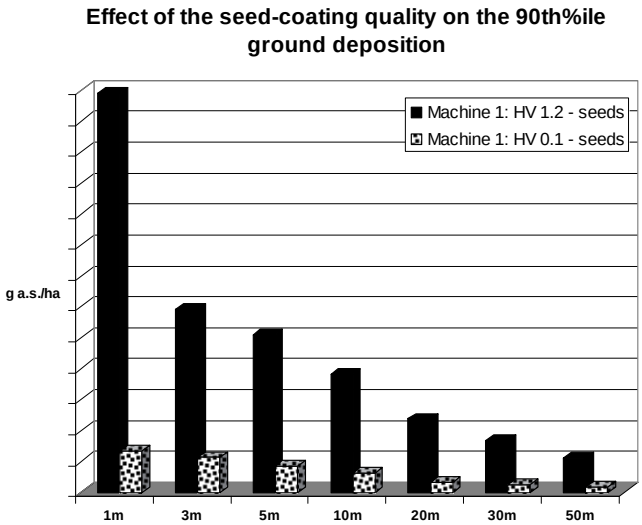


Figure 10 Effect of seed-treatment quality on off-crop ground deposition. Machine 1 is an unmodified reference machine.

The comparison of the corn sowing equipment which was tested with HV 1.2 – seeds, showed that all modified vacuum-pneumatic corn drilling machines along with the mechanical corn drilling machine (i.e. no air assistance) and the corn drilling machine which is operated with compressed air, performed in a comparable way, leading to a significant drift reduction compared to an unmodified vacuum-pneumatic corn drilling machine with an air-stream release directly from the fan (i.e. from one single outlet) (Figure 11).

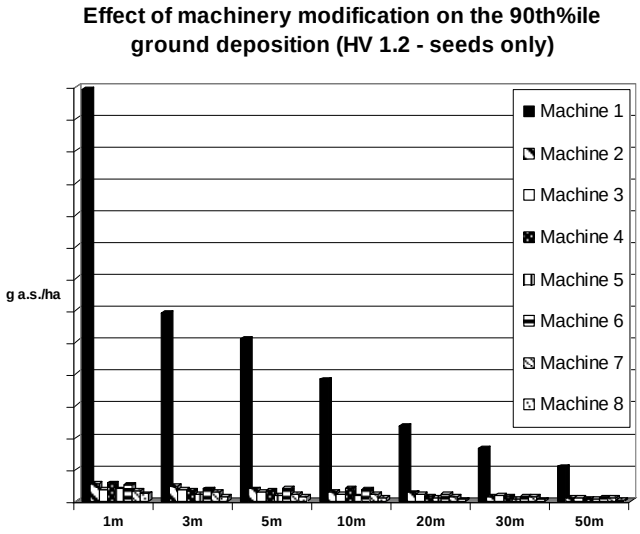


Figure 11 Effect of machinery modification on off-crop ground deposition. Machine 1 is an unmodified reference machine, machines 2-8 are all low-drift machinery

This significant drift reduction became obvious for both, ground deposition and atmospheric drift (Figure 12).

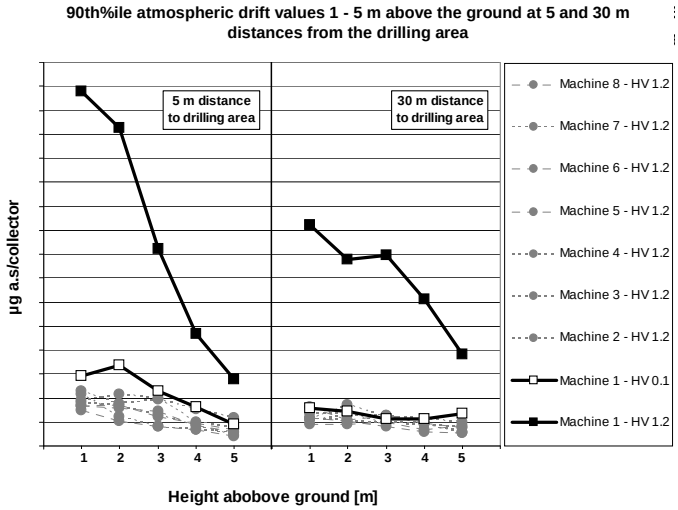


Figure 12 Effect of machinery modification and seed-coating quality on airborne dust. Machine 1 is an unmodified reference machine, machines 2-8 are all low-drift machinery

Although there were variable weather conditions within the 24-hours post-drilling periods during the investigation of the different machinery and seed-coating qualities, the obtained data concerning secondary drift processes show a consistent picture: Secondary drift processes (i.e. the downwind transport of dislodged dust particles deposited during the drilling operation of the soil surface) takes place, if at all, in a negligible extent that it can hardly be detected (Figure 13).

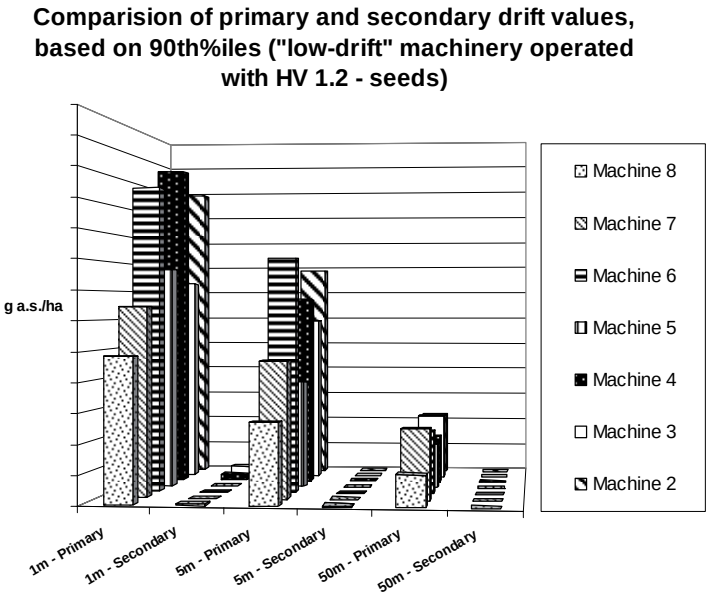


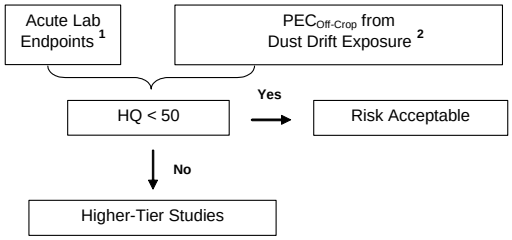
Figure 13 Comparison of primary drift (i.e. dust-drift during and immediately after drilling) and secondary drift (i.e. dust particles dislodged from the drilling area and transported downwind within a 24 h period after end of drilling); machine 2-8: low-drift machinery

Conclusions from the drift field trial: Overall, it could be demonstrated that all modifications mounted to existing vacuum-pneumatic sowing equipment - which all followed the same principal approach (see: Modified vacuum-pneumatic drilling machines) - allow for a successful implementation of low-drift technology, which proved itself to be as effective as e.g. the low-drift technology of a mechanical sowing machine which operated without any air assistance.

Moreover, the effectiveness in terms of a significantly reduced off-crop exposure achieved by the use of the tested modification kits as well as of further modification kits, has been additionally confirmed by independent tests of the competent German Federal authority (JKI). The current status of officially approved low-drift technology for commercial vacuum-pneumatic corn sowing equipment can be found on the webpage of the JKI (http://www.jki.bund.de/); the confirmed drift-reduction amounts to 90% compared to not-modified equipment.

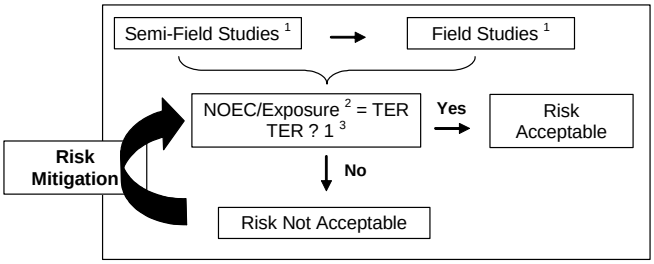
Honey bee risk assessment

IVA Dust risk assessment proposal for bees: The proposed risk assessment scheme outlined in the following was corroborated by an expert working group of the German Industrieverband Agrar (IVA, the association of the crop protection and fertilizer industry in Germany) (Figures 14, 15).



[1] Standard oral and contact acute study results; [2] PEC from off-crop dust exposure including mitigation

Figure 14 Tier 1 honey bee risk assessment scheme for dust exposure



[1] Acute as well as brood effects should be assessed (studies with spray application, potentially studies with dust application); [2] Including refined exposure assessment; [3] TER threshold value depends on available data

Figure 15 Higher-tier honey bee risk assessment for dust exposure

In a Tier 1 screening approach, a hazard quotient is calculated in order to identify those compounds which require higher-tier assessment concerning their risk to honey bees under relevant use conditions. If the hazard quotient, (HQ), based on the lowest toxicity endpoint under standardised laboratory conditions in combination with realistic worst-case exposure to be expected in the off-crop area, is below the conservative threshold value, no further activities are considered necessary. If the HQ is above the trigger value of 50 higher tier studies are required. In the higher tier risk assessment, a TER (Toxicity-Exposure Ratio) approach is applied and exposure data are compared with the results from tunnel or field studies where bees were exposed to the compound under consideration. Tentatively, studies with spray formulations containing the same active compound as the evaluated seed-dressing formulation is considered appropriate for this step of the risk assessment.

Exemplary bee risk assessment for exposure to clothianidin via dust during corn drilling (Bayer CropScience): Following the IVA honey bee risk assessment proposal outlined above, the rate of clothianidin [g a.s./ha] which has not induced increased mortality in a honey bee semi-field cage study, where clothianidin was sprayed into a full-flowering and bee-attractive crop during honey bees were actively foraging (unpublished GLP study data), is compared to the 90th%ile of the field-measured clothianidin exposure values in the off-crop area (ground deposition, 1m distance directly adjacent to the corn drilling area), the following TER value for clothianidin is calculated under consideration of the following parameters:

- a seed loading of 1.25 mg clothianidin a.s./kernel (Poncho Pro®),
- a Heubach dust abrasion value of ≈1.3 g dust / 100,000 corn seeds, and
- modified vacuum-pneumatic corn drilling equipment, mechanical corn drilling equipment or corn drilling equipment which operates with compressed air,

Comparing the toxicity and the exposure value as outlined above, the resulting TER-value is 6. From this TER figure, it can be concluded that it is unlikely that there is an unacceptable risk for honey bees from abraded clothianidin deposits associated with the aforementioned seed-coating quality and machinery parameters. The margin of safety can be further improved by an enhanced seed-coating quality.

Final conclusions and outlook

Substantial work has been undertaken to investigate the causal factors that constituted the bee incident in the Upper Rhine Valley in 2008. Intensive activities were dedicated to develop optimizations in the areas which were identified as key factors for appropriate risk mitigation for seed treatments, i.e. seed-dressing quality and drilling technology. It was demonstrated in comprehensive field studies under realistic conditions that the developed mitigations measures work efficiently.

Therefore it can be concluded that by implementation of the outlined optimizations, the exposure of bees to dusts from seed-coating products during the drilling process can be minimized by orders of magnitude, and that a bee-safe use of insecticidal seed-dressing products can be ensured.

Acknowledgements

We thank all those who co-operated with us in the investigation of the incident and in the development of risk mitigation measures and technical improvements. In particular, we would like to mention:
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Spring honey bee losses in Italy

Laura Bortolotti[1], Anna Gloria Sabatini[1], Franco Mutinelli[2], Mario Astuti[3], Antonio Lavazza[4], Roberto Piro[4], Donato Tesoriero[1], Piotr Medrzycki[1], Fabio Sgolastra[5], Claudio Porrini[5]

[1]CRA–Unità di Ricerca di Apicoltura e Bachicoltura, Via di Saliceto 180, Bologna (Italy), Tel. +39 051 353103, Fax +39 051 356361, e-mail: laura.bortolotti@entecra.it;
[2]Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro (PD) (Italy)
[3]Direzione Generale Sanità Regione Lombardia, Milano (Italy)
[4]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia (Italy) [5]Dipartimento di Scienze e Tecnologie Agroambientali (DiSTA), University of Bologna (Italy)

Abstract

Background: During last years several cases of bee losses have been reported during the period of corn sowing in different European countries. In Italy an institutional system for bee losses survey does not exist and therefore some Italian regions decided to organise an official network to collect data and analyse dead bee samples.

Results: Collected data indicate that the higher number of bee losses events occurred in intensively cultivated flat areas, located in the North of Italy, mainly during or after corn sowing. The chemical analyses of dead bees revealed the presence of three neonicotinoid residues: imidacloprid was found in 25.7% of the sample,

thiamethoxam in 2.8%, clothianidin in 25.7%, both imidacloprid and thiamethoxam in 4.7%. The visual examination and the virological analyses excluded pathological causes.

Conclusion: The spatial and temporal correlation between hive damages and corn sowing and the presence of residues of active ingredients used for seed dressing (imidacloprid, thiamethoxam and clothianidin) in almost half of the samples confirms the connection between spring mortality and the sowing of corn seed dressed with neonicotinoids.

Keywords: honeybee mortality, neonicotinoids, seed dressing, corn sowing, dust dispersion.

Introduction

During last years several outbreaks of honeybee losses have been reported all over Europe and in others countries worldwide. Recently these phenomena became extremely worrying. According to the last researches, the most likely risk factors are bee diseases, agrochemical treatments, poor beekeeping management and climatic changes. These factors can act singularly or simultaneously and can vary depending upon the local circumstances. Among them, the agrochemical treatments performed during spring-summer in intensively cultivated areas seem to have a great impact.

Already in 1999 Italian researchers noted that many reports from beekeepers affected by hive losses coincided with the period of corn sowing and hypothesized that the cause could be dust dispersion from drilling machine during sowing operations of dressed corn seeds. Further investigations demonstrated that a loss of active ingredient (a.i.) through the fan drain of pneumatic seed drills during corn sowing can actually occur [1] and that after the sowing operations flower and grass samples collected near the corn fields are contaminated by the a.i. imidacloprid.[2] The experiments regarded Gaucho® dressed corn seeds.

Bee losses survey is taking place in many European countries including Italy, where it is however not yet well enough organised. At present and waiting for the activation of a national monitoring network, Italian beekeepers can send their reports on hive damages through a specially provided questionnaire, published on the main apicultural magazines and web sites.

The reports in 2008 spring during the period of corn sowing increased exceptionally. In Table 1 the number of reported hive damages in 5 Italian regions is summarized. The total number of affected hives was 6328 and the number of beekeepers 185. These data probably underestimate the total damage, because in Italy beekeepers are not used to report hive damages to the public authorities. For the same period the Italian institution for the surveillance of honey market (Osservatorio Nazionale della Produzione e del Mercato del Miele) estimated a loss of 50,000 hives (http://www.osservatoriomiele.org/2_rapporto2008.htm).

Table 1 Number of reported hive damages during spring 2008 in 5 Italian regions.

Region	n° affected hives	n° affected beekeepers
Lombardy	1513	40
Piedmont	1167	8
Emilia-Romagna	187	7
Veneto and Trentino	1000	20
Friuli Venezia Giulia	2461	110
Total	**6328**	**185**

Aim of the present research is to demonstrate the correlation between colony losses in spring 2008 and the sowing of corn seeds dressed with neonicotinoids.

Experimental methods

In 2008 spring two regions of North Italy (Lombardy and Veneto) decided to organise an institutional network. When beekeepers noted a damage to their bees they had to report it to the local Veterinary Authority and fill in a questionnaire, then the veterinarian should inspect the apiaries and collect samples of dead bees and pollen from surrounding vegetation. Samples were sent to the Istituto Zooprofilattico

Sperimentale of Brescia for the analysis of pathogens and to the CRA-Unità di Ricerca di Apicoltura e Bachicoltura of Bologna for the analysis of neonicotinoid residues (imidacloprid, thiamethoxam, clothianidin).[3,4]

Results

The results of the residue analysis for samples collected in Lombardy and Veneto are summarised in Table 2. A total of 105 dead bee samples were analysed (65 from Lombardy and 40 from Veneto) and 4 samples of pollen from surrounding vegetation in Lombardy. Several samples resulted positive to imidacloprid (25.7%), thiamethoxam (2.8%) and clothianidin (25.7%) and also to both imidacloprid and thiamethoxam (4.7%). Three out of four pollen samples resulted positive to imidacloprid, one also to clothianidin.

Table 2 Results of the analysis of samples collected in 2008 spring in Lombardy and Veneto.

	Lombardy		**Veneto**		**Total**	
	Number	**%**	**Number**	**%**	**Number**	**%**
Analysed samples	69		40		**109**	
Dead bee samples	65		40		**105**	
Positive dead bee samples	30	46.1	22	55.0	**52**	**49.5**
Dead bee samples positive to imidacloprid	19	29.2	8	20.0	**27**	**25.7**
Dead bee samples positive to thiamethoxam	2	3.0	1	2.5	**3**	**2.8**
Dead bee samples positive to clothianidin	13	20.0	14	35.0	**27**	**25.7**
Dead bee samples positive to fipronil	0	0	0	0	**0**	**0**
Dead bee samples positive to both imidacloprid and clothianidin	4	6.1	1	2.5	**5**	**4.7**
Pollen samples	4				**4**	
Positive pollen samples	3	75.0			**3**	**75.0**
Pollen samples positive to imidacloprid	3	75.0			**3**	**75.0**
Pollen samples positive to thiamethoxam	0	0			**0**	**0**
Pollen samples positive to clothianidin	1	25.0			**1**	**25.0**
Pollen samples positive to both imidacloprid and clothianidin	1	25.0			**1**	**25.0**

The concentrations of a.i. found in dead bee samples (52 positive samples) ranged from 1.01 to 240.6 ng/g for imidacloprid, from 3.67 to 39.2 ng/g for clothianidin and from 24.8 to 138 ng/g for thiamethoxam. The concentration of a.i. found in pollen samples (3 positive samples) ranged from 7.3 of clothianidin to 311.45 ng/g of imidacloprid.

The inspection carried out by the Veterinary Services and the results of virological analysis excluded any pathological cause.

In Table 3, we report the data of the questionnaire completed by beekeepers and veterinarians, related to the 65 apiaries affected in Lombardy, corresponding to 1513 hives. All the reports came from cultivated areas, located mostly in the plain area; the main surrounding crop was corn and in 96.2% of cases the damage occurred during or after corn sowing. Affected hives had rich brood combs and abundant stores and the foraging activity was intense. In 91% of the affected hives an anomalous behaviour of workers was observed, consistent with those reported after intoxication with neonicotinoids.[5,6]

Table 3 – Results of the questionnaire filled in by beekeepers in 2008 spring in Lombardy region.

Number of questionnaires	**65**
Number of affected hive in each apiary	from a minimum of **3** to a maximum of **170**
Hives types	**93%** sedentary; **7%**migratory
Range of dead bees for each hive	from few hundreds to many thousands (**up to 15,000-20,000**)
Areas	**69%** plain; **20%** hills; **11%** mixed areas
Main surrounding crop	**96%** corn; **55%** wheat; **33%** meadows
Period	**96.2%** of cases during or after corn sowing
Stores	Presence of rich brood combs and abundant honey and pollen stores
Foraging activity	Intense at the time of sowing (presence of foragers with pollen loads in the **95.8%** of cases)
Worker behaviour	Anomalous in **91%** of cases: rolling **71.4%;** disorientation **57.4%;** aggressiveness **23.8%;** incapability to enter the hive **52.3%.**

Discussion and conclusions

The results of the study allow some relevant conclusions:

- there is a spatial and temporal correlation between hive damages and corn sowing;
- the presence of residues of a.i. used for seed dressing (imidacloprid, thiamethoxam and clothianidin) in almost half of the samples confirms the relationship between spring mortality and the sowing of corn seed dressed with neonicotinoids.

The fact that half of the analysed samples did not contain residues is not enough to exclude the responsibility of neonicotinoids in hive damages. Many factors can influence the presence of residues and their level: the way of exposure of bees to the a.i., that can be direct during corn sowing or indirect via pollen and nectar of surrounding flora; dead bee samples could have been collected with some delay after intoxication or could have not been properly stored with a consequent degradation of the a.i.

Following these evidences, on 17th of September 2008 the Italian Government decided the precautionary suspension of use of all the four a.i. registered for seed dressing - imidacloprid, thiamethoxam, clothianidin and fipronil - although the latter was never found in dead bee samples.

The future implementation of an Italian national bee monitoring network (APENET), which hopefully will be implemented by 2009, will certainly contribute to the knowledge of the extent and causes of this phenomenon. [7]

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Sprayed and seed dressed pesticides in pollen, nectar and honey of oilseed rape

Klaus Wallner

University Hohenheim, Apiculture Institute, D-70593 Stuttgart
E-mail: Klaus.Wallner@uni-hohenheim.de

Introduction

Oilseed rape is almost exclusively produced as an intense cultivation. Seeds are treated before sowing with systemic insecticides, nowadays primarily with neonicotinoids. In the blooming period, sprays against fungi (*Sclerotinia sclero)* or pests (e.g. *Ceutorhynchus assimilis*) with different non hazardous pesticides are common. These substances are known to reach pollen and nectar. Contaminants in food sources are actually discussed as sublethal factors influencing colony health. Residues are adverse for the image of honey.

Experimental

In a study to quantitative study the presence of residues in nectar, pollen and honey conditions, two fungicides were sprayed into an 8 ha blooming cultivation in accordance with normal agricultural practice in Germany. Over a 7 day period, residues of the seed dressed insecticide and the sprayed fungicides were measured in the pollen and nectar loads of returning foragers. Unripe honey from combs and extracted honeys were analyzed.

16th April 2007: Application of the fungicides Cantus® (boscalid, 500g a.i./kg), 0,5 kg/ha) and Proline® (prothioconazol, 250g a.i./kg, 0,7 kg/ha) in an 8 ha oilseed rape field (variety *Smart)*, seed dressed with the insecticide Elado Premiumbeize® (clothianidin a.o.). Both fungicides act systemically and can be combined with non hazardous pyrethroids or neonicotinoids insecticides. The fungicides were sprayed in combined application with 250 l water per ha.

Two apiaries (2 respectivally 7 colonies) at 200 m distance to the sprayed oilseed rape field were used for the experiments.

Returning foragers were caught at the hive entrance with a special vacuum cleaner. The bees were immediately shock frozen with carbon dioxide snow and stored at -20°C until preparation. Thus, starting from the day before application, at least three series with around 100-150 bees were collected per day over a 7 day period

In the lab, the pollen loads and the collected nectar of the honey sacs were prepared separately for each trapped group of forager bees. The pollen loads were sorted by color and the origin was checked under the microscope. Only oilseed rape pollen was used for further analysis. In total 22 pooled pollen and 22 pooled nectar samples were prepared with an adapted QuEChERS-multi method and analyzed with tandem LC-MS/MS. The quantitation limits for the different substances in the analysis were as follows:

- Boscalid in pollen, nectar and honey: 0,001 mg/kg
- Prothioconazol in nectar und honey: 0,001 mg/kg
- Prothioconazol in pollen: 0,010 mg/kg
- Clothianidin in pollen, nectar und honey: 0,001 mg/kg

Results

Pesticides in pollen loads

Prothioconazol and clothianidin were not detected in the pollen loads of returning bees over the whole period.

Boscalid was detected in all 22 series. At the day of application the detected average boscalid value in the pollen loads was 13.9 mg/kg and at the following day 26.2 mg/kg. At the second day the contamination decreased to 4.7 mg/kg and stayed on this level the following days. At 7 days after the application, boscalid was still measured at levels around 3 mg/kg.

Residues in nectar

All three pesticides were detected in the nectar in the honey sac loads over the 7 day period. Boscalid and prothioconazol residues were in high ppb-levels after the application (1.43 mg/kg respectivally 0.69 mg/kg). The values decreased to 0.13 respectively 0.06 mg/kg the following day and for both substances to 0.017 mg/kg the second day. After 7 days the boscalid value reached 0.025 mg/kg and the prothioconazol 0.009 mg/kg. The clothianidin values moved between 0.001-0.003 mg/kg and were always near the limit of quantitation. Clothianidin acted like an internal standard and showed that the forager bees intensely used the treated oilseed rape field.

Conclusions

Spraying of boscalid in oil seed rape according to normal agricultural practice in Germany causes residues in pollen (above the German MRL), nectar and honey. Prothioconazol was detected in nectar and honey. Due to matrix effects and irreversible adsorption effects, this pesticide is not detectable in pollen. Its residual behavior is still unclear. Clothianidin migrates from the plant into nectar in low traces near the LoQ. Even with low quantitation limits (0.001 mg/kg), this insecticide was not detected in pollen or honey. The fungicide spray application leads to appreciably higher residues in the bee products than the seed treatment, particularly in the time after the application. Systemic properties of the three substances induce the contamination of pollen and nectar over a prolonged time. The hydrophilic character of the fungicides may lead to relatively low residues in rape oil, but to relative high residues in honey. Pollen traps should be closed at least for the first few days after spray applications.

Colony losses – interactions of plant protection products and other factors

Martina Wehling*, Werner von der Ohe, Dietrich Brasse, Rolf Forster

*LAVES Institut für Bienenkunde Celle, Herzogin-Eleonore-Allee 5, 29221 Celle

Abstract

In recent years repeated colony losses occurred in Germany. Besides Varroosis many other possible causes like bee diseases, nutrition supply as well as effects of pesticides have been discussed.

A chronic feeding study was conducted to find indications to what extent negative effects of pesticides in sub-lethal doses can be discerned from effects of other stressors (pathogens, drugs, mix of plant protection products, malnutrition of proteins) or any interactions or coactions.

In a screening programme effects of chronic dietary exposure to sub-lethal doses of the insecticide imidacloprid were studied in honeybees under stress of another potential stressor (*Varroa destructor*, *Nosema apis*, drugs, lack of pollen supply). The results confirm a chronic oral toxicity of imidacloprid at concentrations which in several previous studies have been reported to be toxic to bees (100 ppb). However, no indications were found for significant differences in sensitivity to imidacloprid between bees under other stressors and control bees.

Results confirm previous findings that optimal of protein supply can soften negative effects of stressors. In addition it became apparent that bees from different colonies of the same apiary which were fed in parallel varied in sensitivity.

A semi-field experiment was conducted to asses the risks of mixing plant protection products by simulating commercial applications during blooming on bee colonies foraging in commercial seed dressed rape with potential residues in nectar and pollen.

No adverse effects on mortality or on development of exposed bee colonies had been found when bees foraged on rape of dressed seeds and plants were sprayed with one single plant protection product (pyrethroid resp. azol-fungicide) or in combination (tank mix pyrethroid plus azol-fungicide).

From the findings of chronic feeding tests and semi-field test it can be concluded that imidacloprid used as standard seed dressing formulation will pose no risks to honeybees.

List of participants

Anne Alix
AFSSA
10 Rue Pierre Curie
94704 Maison Alfort
France
a.alix@afssa.fr

Pierrick Aupinel
INRA Centre Poitou Charentes domaine du Magneraud
17700 Surgères
France
pierrick.aupinel@magneraud.inra.fr

Cedric Bachelier
AFSSA
10 Rue Pierre Curie
94704 Maison Alfort
France
c.bachelier@afssa.fr

Frank Bakker
Mitox Consultants
Kruislaan 406
1098 SM Amsterdam
The Netherlands
frank.bakker@mitox.org

Katie Barrett
HLS
Woolley Road, Alconbury
PE28 4HS Huntingdon
UK
barrettK@ukorg.huntindon.com

Anja Bartels
AGES
Spargelfeldstrasse 191
A 1226 Vienna
Austria
anja.bartels@ages.at

Markus Barth
Biochem agrar GmbH
Kupferstr 6
4827 Gerichshain
Germany
markus.barth@biochemagrar.de

Roland Becker
BASF SE
Carl Bosch Str.
67117 Limburgerhof
Germany
roland.becker@basf.com

Sigrun Bocksch
Eurofins-GAB
Eutinger Str 24
75223 Niefern-Oschelbronn
Germany
sigrun.boksch@eurofins-gab.com

Laura Bortolloti
CRA
Via di Saliceto 80
40128 Bologna
Italy
laura.bortollotti@entecra.it

Dietrich Brasse
RifCon
Lutzowstr 1
38102 Braunschweig
Germany
dietrich.brasse@rifcon.de

Tricia Brobyn
PSD
3 Peasholme Green, King's Pool
YO1 7PX York
UK
tricia.brobyn@psd.hse.gsi.gov.uk

Etienne Bruneau
CARI asbl
4 Place Croix de Sud
B1348 Louvain la Neuve
Belgium
bruneau@cari.be

Dace Bumane
State Plant Protection Service
Lielvardes Street 36/38
LV-1006 Riga Latvia
dace.bumane@vaad.gov.lv

Marco Candolfi
BASF SE
Carl Bosch Str.
67117 Limburgerhof
Germany
marco.candolfi@basf.com

Yves Coconnier
Staphyt
23 Route de Moeuvres
62860 Inchy en Artois
France
ycoconnier@staphyt.fr

Cristian Constantinescu
Apimondia Romania
B-dul Ficusului 40, sector 1
13975 Bucuresti
Romania
cconst@apimondiafoundation.org

Mike Coulson
Syngenta Crop Protection
Jealotts Hill Int Res Stn
RG42 6EY Bracknell
UK
mike.coulson@syngenta.com

Valeria Croce
Chemservice
Via F.lli Beltrami 15
20026 Novate Milanese
Italy
v.croce@chemservice.it

Gaelle Cure
Bayer CropScience France
16 Rue Jean-Marie Leclair -CP-310
69337 Lyon Cedex 09
France
gaelle.cure@bayercropscience.com

Axel Decourtye
ACTA
Institut C. Bourgelat, 1 Avenue C Bourgelat
68280Marcy l'Etoile
France
axel.decourtye@acta.assoc.fr

Axel Dinter
DuPont de Nemours (Deutschland)
DuPont Str 1
61352 Bad Homburg vdH
Germany
axel.dinter@deu.dupont.com

Mihaela Dogaru
Syngenta Agro SRL Romania
Victoria Park Building 3, Ploiesti Road 73-81
13685 Bucuresti
Romania
mihaela.dogaru@syngenta.com

Sonica Drosu
Research Institute Plant Protection
Bd Ion Ionescu de la Brad 8
13813 Bucuresti
Romania
Sonicadrosu@yahoo.com

Sophie Duchard
AFSSA
10 Rue Pierre Curie
94704Maison Alfort
France
s.duchard@afssa.fr

Rolf Forster
BVL
Messeweg 11/12
38104 Braunschweig
Germany
rolf.forster@bvl.bund.de

Annie Forster
APC
118 Old Exeter Road
PL19 0JB Tavistock
UK
annie.forster@btinternet.com

Hervé Giffard
Testapi
464 Sarré
49350 Gennes
France
giffard.testapi@wanadoo.fr

Fabrice Godet
Bayer CropScience France
16 Rue Jean-Marie Leclair -CP-310
69337 Lyon Cedex 09
France
fabrice.godet@bayercropscience.com

Christian Grenouillet
Monsanto France
Europac du Chêne, 1 Rue Jacques Monod
69673Brom
France
christian.grenouillet@monsanto.com

Sabine Hecht-Rost
Eurofins-GAB
Eutinger Str 24
75223 Niefern-Oschelbronn
Germany
sabine.hecht-rost@eurofins-gab.com

Julie Howarth
PSD
3 Peasholme Green, King's Pool
YO1 7PX York
UK
julie.howarth@psd.hse.gsi.gov.uk

Martina Janke
LAVES
Herzogin-Eleonore-Allee 5,
29221 Celle
Germany
martina.janke@laves.niedersachsen.de

Lukas Jeker
RCC
Zegliweg 1
4452 Itingen
Switzerland
jeker.lukas@rcc.ch

Claudia Jilesen
Plant Protection Service
P O Box 9102
6700 HC Wageningen
The Netherlands
c.j.t.j.jilesen@minlnv.nl

Reet Karise
Estonian University of Life Sciences
Kreutzwaldi 1
51014 Tartu
Estonia
reet.karise@emu.ee

Janine Kievits
Inter-Environment Wallonie
6 Boulevard du Nord
B-5000 Namur
Belgium
kievits@cari.be

Jean-Michel Laporte
Syngenta Agro France
78286 Guyancourt
France
jean-michel.laporte@syngenta.com

Gavin Lewis
JSC International
Simpson House, Windsor Court, Clarence Drive
HG1 2PE Harrogate
UK
gavin.lewis@jsci.co.uk

Jose-Anne Lortsch
Syndicat National d'Apiculture
5 Rue de Copenhague
75008 Paris
France
jalsna@orange.fr

Johannes Luckmann
Rifcon GmbH
Im Neuenheimer Feld 517
69120 Heidelberg
Germany
johannes.lueckmann@rifcon.de

Franck Marolleau
AFSSA
10 Rue Pierre Curie
94704 Maison Alfort
France
f.marolleau@afssa.fr

Cristina Mateescu
Inst Apicultural Research & Development
B-dul Ficusului 42, sector 1
13975 Bucuresti
Romania
cristina.mateescu@icdapiculture.com

Christian Maus
Bayer CropScience AG
Alfred Nobel Strasse 50
40789 Monheim
Germany
christian.maus@bayercropscience.com

Piotr Medrzycki
CRA
Via di Saliceto 80
40128 Bologna
Italy
piotr@inapicoltura.org

Slobodan Milenkovic
Serbian Beekeeping Association
Molerova str 13
11000 Belgrade
Serbia
spos@sezampro.yu

Carmen Mincea
Research Institute Plant Protection
Bd Ion Ionescu de la Brad 8
13813 Bucuresti
Romania
carmenmincea@yahoo.com

Marija Molovrh
Slovenian Institute for Hop Research
Cesta Zalskega Tabora 2
3310 Slovenia
Slovenia
marija.malovrh@ihps.si

Veerle Mommaerts
VUB
Pleinlaan 2
1050 Brussels
Belgium
veerle.mommaerts@vub.ac.be

Jacob Peter van Praagh
DBIB/EPBA
Hassellstr 23
29223 Celle
Germany
jph@tiho-hannover.de

Ion Mutafa
Sc Bayer SRL
9-9a D Pompei Ave, Iride Centre, Building 20
20335 Bucuresti
Romania
ion.mutafa@bayercropscience.com

Alexandru Precup
Syngenta Agro SRL Romania
Victoria Park Building 3, Ploiesti Road 73-81
13685 Bucuresti
Romania
alexandru.precup@syngenta.com

Karin Nienstedt
EFSA
Largo N Palli 5a
43100 Parma
Italy
karin.nienstedt@efsa.europa.eu

Dunja Przygoda
Bayer CropScience
Alfred Nobel Str 50
40789 Monheim
Germany
dunja.przygoda@bayercropscience.com

Florin Oancea
Research Institute Plant Protection
Bd Ion Ionescu de la Brad 8
13813 Bucuresti
Romania
Florino@ping.ro

Pascal Radix
Bayer CropScience
Alfred Nobel Str 50
40789 Monheim
Germany
pascal.radix@bayerbropscience.com

Pieter Oomen
Plant Protection Service
POBox 9102
6700 HC Wageningen
The Netherlands
p.a.oomen@minlnv.nl

Marit Randall
Norwegian Food Safety Authority
RK OAO PO Box 3
1431 As
Norway
marit.randall@mattilsynet.no

Vincent Ortoli
Staphyt
23 Route de Moeuvres
62860 Inchy en Artois
France
vortoli@stahyt.fr

Hans-Ulrich Rexer
Eurofins-GAB
Eutinger Str 24
75223 Niefern-Oschelbronn
Germany
hans-ulrich.rexer@eurofins-gab.com

Edward Pilling
Syngenta Crop Protection
Jealotts Hill Int Res Stn
RG42 6EY Bracknell
UK
ed.pilling@syngenta.com

Natalie Ruddle
Syngenta Crop Protection
Jealotts Hill Int Res Stn
RG42 6EY Bracknell
UK
natalie.ruddle@syngenta.com

Jens Pistorius
JKI
Messeweg 11/12
38104 Braunschweig
Germany
jens.pistorius@jki.bund.de

Stephan Schmitzer
Ibacon GmbH
Arheilgerweg 17
6438 Rossdorf
Germany
stephan.schmitzer@ibacon.com

Guy Smagghe
Ghent University
Coupure Links 653
B-9000 Ghent
Belgium
guy.smagghe@ugent.be

Sjef van der Steen
PRI
Postbus 16
6700 AA Wageningen
The Netherlands
sjef.vandersteen@wur.nl

Guido Sterk
Biobest
Ilse Velden 48
B-2260 Westerlo
Belgium
info@biobest.be

John Stevenson
16 Old Rectory Close
Harpenden
AL5 2UD Herts
UK
john@no16orc.fsnet.co.uk

Helen Thompson
CSL
Sand Hutton
YO41 1LZ York
UK
h.thompson@csl.gov.uk

Ingo Tornier
Eurofins-GAB
Eutinger Str 24
75223 Niefern-Oschelbronn
Germany
ingo.tournier@eurofins-gab.com

Janis Trops
Latvian Beekeeping Association
Elektribas Street 6
LV-3001 Jelgava
Latvia
janis.trops@strops.lv

Rolf TulstrupThueuerkauf
Danish Beekeepers Association
Mollevej 15
4140 Borup
Denmark
rt@bioavl.dk

Christine Vergnet
AFSSA
10 Rue Pierre Curie
94704 Maison Alfort
France
c.vergnet@afssa.fr

Claude Vergnet
AFSSA
10 Rue Pierre Curie
94704 Maison Alfort
France
cl.vergnet@afssa.fr

Peter van Vliet
CTGB
Stadsbrink 5
6707 AA Wageningen
The Netherlands
peter.vvliet@ctgb.nl

Klaus Wallner
University Hohenheim
August von Hartmann Strasse 13
D-70593 Stuttgart
Germany
bienewa@uni-hohenheim.de

Jacoba Wassenberg
CTGB
Stadsbrink 5
6707 AA Wageningen
The Netherlands
jacoba.wassenberg@ctgb.nl

Selwyn Wilkins
National Bee Unit
CSL, Sand Hutton
YO41 1LZ York
UK
p.wilkins@csl.gov.uk

Vlasta Zlof
EPPO
1 Rue Le Nôtre
75016 Paris
France
zlof@EPPO.fr

Glossary

a.i.	active ingredient
a.s.	active substance
ABPV	Acute Bee Paralysis Virus
AFSSA	French Agency on the Safety of Food, F
APENET	Italian National Bee Monitoring Network, I
ARfD	Acute Reference Dose
BPG	Bee Protection Group
BVL	Federal Office of Consumer Protection and Food Safety, D
CCD	Colony Collapse Disorder
CEB	Commission des Essais Biologiques, F
CSL	Central Science Laboratory, UK
DAA	Days After Application
DWV	Deformed Wing Virus
EC	European Commission
EFSA	European Food Safety Authority
EPPO	European and Mediterranean Organisation for Plant Protection
ESI	Elektro Spray Ionization
EU	European Union
FERA	Food and Environment Research Agency, UK
GAP	Good Agricultural Practice
GLP	Good Laboratory Practice
HPG	Hypopharyngeal Glands
HPLC	High Performance Liquid Chromatography
HQ	Hazard Quotient
IAPV	Israel Acute Paralysis Virus
ICP-BR	International Commission for Plant-Bee Relationships
IGR	Insect Growth Regulator
IPM	Integrated Pest Management
IUBS	International Union of Biological Sciences
IVA	German Industrieverband Agrar, D
JKI	Julius Kühn Institut, D
KBV	Kashmere Bee Virus
LD_{50}	Lethal Dose 50
LoD	Limit of Detection
LOEC	Lowest Observed Effect Concentration
LoQ	Limit of Quantification
LTZ	Landwirtschaftliches Technologiezentrum Augustenberg, D
MCA	Microbiological Control Agents
MFO	Mixed Function Oxydase
MFRC	Maximum Field Recommended Concentration
MLR	Ministry of Food and Rural Land, D
MRL	Maximum Residue Limits .
MS	Mass Spectrometry
NOEC	No Observed Effect Concentration
NOEL	No Observed Effect Level
OECD	Organisation for Economic Cooperation and Development
OSR	Oil Seed Rape
PEC	Predicted Environmental Concentration
PNEC	Predicted No Effect Concentration
P_{ow}	Partition Coefficient Octanol/Water
PPP	Plant Protection Product
PPS	Plant Protection Service, NL
RH	Relative Humidity
RPF	Regional Councils of Freiburg, D
RPS	Regional Councils of Stuttgart, D
SANCO	Directorate General Health and Consumers of the EC
SBV	Sacbrood Bee Virus
TER	Toxicity Exposure Ratio
TMD	Total Maximum Daily Intake

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Informationszentrum und Bibliothek
Julius Kühn-Institut, Bundesforschungsinstitut für Kulturpflanzen
Königin-Luise-Straße 19
D-14195 Berlin, Germany
E-Mail: ib@jki.bund.de

Hazards of pesticides to bees

Honeybees are cherished by the public, and everybody will agree that their well-being is important. The fate of honeybees in Europe and worldwide attracts extensive public attention, even of politicians and in the European Parliament.

Following reports of serious poisoning of honeybees by pesticides across Europe in 1978 and 1979, agricultural scientists in the Netherlands supported by colleagues in France, Germany, Switzerland and England set up the ICP-BR Bee Protection Group. The first meeting of scientists from government agencies, industry and universities was held in Wageningen in 1980. Their objective was, and remains to ensure the safety of honeybees and other bee species in agricultural crops and to ensure that they are not harmed by the approved use of plant protection chemicals.

Since 1980 poisoning of honeybees has been greatly reduced thanks in no small part to the work of the Group's members, to better understanding of the reasons for bee poisoning and to the introduction of safer insecticides and modern advances in crop protection techniques.

However in recent years there has been universal concern about a serious worldwide collapse of honeybee colonies, often referred to as Colony Collapse Disorder or Bee Decline. Scientists, including members of the Bee Protection Group are actively searching for the precise reasons and hence for a cure. Bad winter survival, genetic problems, the Varroa mite, diseases and often pesticides were named as culprits.

At the 9th symposium of the Bee Protection Group (York, 2005) several specialist groups were formed to address the most important of these problems. These groups reported in the 10th symposium (Bucharest, 2008) with proposals for better risk assessments for systemic insecticides, better semi-field and field testing and better bee brood testing. These proposals are published for the first time in these proceedings of the Bucharest symposium, together with the reports of many other new developments in the area of protecting honeybees from the undesired effects of pesticides. One particular undesired effect reported here are the incidents caused by dust abrasion from treated seeds in Germany, France, Italy and Slovenia. The proposals resulting from the working groups, and from the discussions and recommendations of the symposium will be processed by EPPO (European and Mediterranean Plant Protection Organisation) to a new environmental risk assessment scheme for plant protection products and honeybees in Europe.
In spite of considerable effort the precise cause of Bee Decline remains obscure, although significant progress has been made recently, and the Bee Protection Group continues to play its part in seeking an early solution. However careful analysis of all the available data shows that pesticides are not the cause.

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