EXTERNAL SCIENTIFIC REPORT

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Chronic oral lethal and sub-lethal toxicities of different binary mixtures of pesticides and contaminants in bees (*Apis mellifera*, *Osmia bicornis* and *Bombus terrestris*)

Centre for Ecology & Hydrology

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Abstract

Chemical hazard assessment for bees generally starts with laboratory testing of acute effects in the honeybee, *Apis mellifera*. Whether acute effects observed in this model species translate to different species and longer exposure periods are key issues for robust hazard assessment. The aim of this study was to develop and trial a series of toxicity tests for testing the prolonged (up to 240 h) exposure of *A. mellifera*, *Bombus terrestris* and *Osmia bicornis* to a range of insecticides (clothianidin, dimethoate, tau-fluvalinate), other pesticides (propiconazole, 2,4-D) and trace metals (cadmium and arsenic) and selected mixtures. Oral toxicity tests for the species were developed from standardised procedures that were modified to account for species ecologies and behaviours. Tests with *A. mellifera* identified that toxicity decreased in the order clothianidin > dimethoate > cadmium > arsenic > tau-fluvalinate > propiconazole >= 2,4-D. This order of sensitivity was broadly consistent for the other two species. The only chemical showing any trend for interspecies variation in sensitivity was tau-fluvalinate. While not toxic to *A. mellifera* at the maximum tested concentration, mortality effects were seen in the other species. Patterns of toxicity showed that LC_{50}s decreased with time. Extension of tests to 240 h and prediction of exposure concentration effects up to a theoretical *Apis* worker bee life-time (720 h) suggest that long-term toxicity may exceed predictions based on short-term tests by an order of magnitude. Mixture tests showed that most commonly tested combinations were additive and non-interactive. Studies with clothianidin and propiconazole did point to a slightly increased toxicity for the neonicotinoid in the presence of the fungicide. For dimethoate and clothianidin in *B. terrestris* and to an extent *O. bicornis,* weak antagonistic interactions were found. These finding suggest that, at least for initial assessment, current mixture models frequently provide a relevant indication of likely joint effect.

Key words: Ecotoxicology, Species sensitivity, Exposure time, DEBtox, toxicokinetic, toxicodynamics

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Summary

Current Organisation for Economic Co-operation (OECD) toxicity test protocols were modified to allow assessment of oral lethal and sub-lethal effects of seven chemicals in *Apis mellifera* , *Bombus terrestris* and *Osmia bicornis* for over 240 h periods. For *O. bicornis* initial tests were characterised by high control mortality. Optimisation of the experimental procedures (especially early feeding and selection) greatly decreased control mortality and improved the robustness of the tests.

The datasets obtained from the tests provide an excellent basis to establish the relative chemical sensitivity between bee species and to identify patterns of single chemical effects in time and mixture effects among different chemical combinations.

The use of toxicity tests to provide an extended exposure of *A. mellifera* to each of the seven test chemical allowed dataset to be generated that examine the effects of exposure time on toxicity (expressed as metric such as the LC_{50}) and for DEBtox modelling from which prediction of sensitivity for exposure time beyond the experimental duration could be made.

Two chemicals, namely 2,4-D and tau-fluvalinate, did not show effects on survival at tested concentrations and propiconazole showed only a partial mortality over 10 days. *Apis mellifera* was most sensitive to the two remaining insecticides (clothianidin > dimethoate >> tau-fluvalinate), followed by the trace metals and then fungicide and herbicide.

Trends for LC_{50} over time calculated from the DEBtox model fits indicated a 25-fold change when exposure was extended from 96 to 720 h for cadmium, the most time-dependent chemical. This change was in the order of 10-fold, and < 10-fold for clothianidin, dimethoate and arsenic. This suggests that extending exposure duration may results in LC_{50} 10-fold lower than those from short-term tests.

The ranking of the comparative toxicity of the test chemicals was broadly similar for each of the three selected bee species. Changes of sensitivity in time for *B. terrestris* and *O. bicornis* were broadly consistent with those observed for *A. mellifera*. The only tested chemical for which a degree of divergence was seen in the estimates of toxicity was for tau-fluvalinate. This chemical showed a higher toxicity to *B. terrestris* and *O. bicornis* than for *A. mellifera*.

Behavioural traits were also tractable to measure. These were more sensitive than measured mortality by a factor of 1.5–2.5, especially for *B. terrestris*. Larval studies provide a further tool to assess toxicity. They showed that survival and final body weight could be effectively measured in a relatively high throughput system.

Effects on mortality in mixture tests were general consistent with the results of the previous single chemical studies. The temporal patterns of toxicity seen are amenable to analysis using the DEBtox model framework giving the potential to identify toxicokinetic and toxicodynamic parameters, as well as no effect concentrations. Two types of mixture study were conducted to analyse potentation and mixture toxicity.

No clear or consistent species or time-dependent potentiation of dimethoate by propiconazole or clothianidin by tau-fluvalinate was seen in across tests and time points. The studies with clothianidin and propiconazole did point to a slightly increased toxicity for the neonicotinoid in the presence of the fungicide. This change was a maximum of approximately two-fold in *O. bicornis*, although lower in *A. mellifera*. The magnitude of such a change does not mirror the orders of magnitude changes previously reported for studies between tau-fluvalinate and sterol biosynthesis inhibiting fungicides.

The five mixture toxicity studies conducted also point to a likely limited degree of interaction between chemicals. The dominant response patterns reflected the expectation of additivity calculated according to either concentration addition, independent action or both. Only for dimethoate and clothianidin in *A. mellifera*, *B. terrestris* and in *O. bicornis* males is there any evidence of an interaction between the

chemicals that causes a deviation for predicted additive effects. This interaction for this binary mixture is predominantly antagonistic. This presence of this antagonistic interaction I for dimethoate and clothianidin mixtures in all three tested species is also weakly supported by DEBtox models which show possible interaction, most clearly in the fit for *B. terrestris*.

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1. Introduction

1.1. Background and Terms of Reference as provided by EFSA

One of the objectives of the European Food Safety Authority (EFSA) Science Strategy (2012-2016) is to broaden risk assessment in a wider integrated manner, to develop harmonised risk assessment methodologies and horizontal scientific approaches and to promote in-house scientific expertise, tightening transversal collaborations across units. In relation to the development of harmonised risk assessment methodologies, areas of priority for EFSA have been identified and include environmental risk assessment and risk assessment of chemical mixtures.

In the field of environmental risk assessment, the EFSA Scientific Panel on Plant Protection Products and their Residues (PPR Panel) has recently published a Scientific Opinion on the science behind the development of a risk assessment of Plant Protection Products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees) (EFSA PPR Panel, 2012a) which considered four key issues: (i) assessment of the acute and chronic effects of plant protection products on bees, including colony survival and development; (ii) estimation of long-term effects due to exposure to low concentrations; (iii) development of a methodology to take into account cumulative and synergistic effects; and (iv) evaluation of the existing validated test protocols and the possible need to develop new protocols, especially to take into account the exposure of bees to pesticides through nectar and pollen. From a horizontal perspective, EFSA set up an internal multidisciplinary Task Force on bee risk assessment coordinated by the Emerging Risks Unit (EMRISK) (M-2012-01514) and involving staff from the Pesticides Unit (PRAS), Animal Health and Welfare Unit (AHAW), Plant Health Unit (PLH), Genetically Modified Organisms Unit (GMO), and the Scientific Assessment Support Unit (SAS), as well as staff from the Communications Directorate. The first output of this Task Force has been published in a report describing an 'Inventory of EFSA's activities on bees' summarising the work carried out at EFSA to date (EFSA, 2012b). The second output of this Task Force, currently under progress, will review and analyse national and international activities carried out outside of EFSA on bee risk assessment, with a view to identifying data gaps and future research needs.

Both the Scientific Opinion of the PPR Panel and the Technical Report of the EFSA Task Force have concluded on specific recommendations regarding research needs. These requirements include acute and chronic toxicological studies for both lethal and sub-lethal effects for a wider range of single and/or multiple pesticides and environmental contaminants (e.g. mycotoxins, heavy metals, etc.) in adults and larvae honeybees, solitary bees and bumble bees both in the laboratory and in the field. With respect to chemical mixtures and potential synergistic effects, such studies will generate dose responses of combined toxicity for environmentally realistic combinations of chemicals in bees. This will then provide a basis to develop models to predict acute and chronic effects on individual bees and populations. However, when testing the effects of chemicals on bees, a single generic test does not provide information on population dynamics because of the complex social life and dynamic behaviour of bee colonies, which can be considered as super-organisms with complex interactions. Therefore, there is a need to explore the feasibility of integrating the results of toxicological studies into population models. In this respect, population models using the Dynamic Energy Budget (DEB) theory systematically incorporates the exposure time to chemical(s) together with the biology of the organisms including life cycle information (feeding, maintenance, growth, development and reproduction).

DEB models can be used to extrapolate toxic effects for single compounds and mixtures, measured at the individual level to meaningful consequences at population level: DEBTOX (Baas et al., 2010; Jager, 2012). DEBTOX models have been applied to the effects of chemical toxicants on survival rate to establish no effect concentrations for a number of organisms in environmental risk assessment; e.g. on juvenile fish growth, Daphnia reproduction, algal population growth, tumour induction and growth

in mammals (Kooijman and Bedaux, 1996; Kooijman et al., 1996; van Leeuwen et al., 2003; Bontje et al., 2009); but their potential to assess bee population dynamics has not yet been explored.

In order to fulfil these recommendations, a first step is to perform a pilot study to test the combined acute and chronic toxicity of chemicals (pesticides and contaminants) in bees (adults and larvae) and generate a state of the art of DEBTOX models for bee populations.

The three specific objectives are as follows:

- Objective 1: test the acute oral lethal toxicity of single and multiple pesticides and contaminants in bees (i.e. in adults and larvae of honeybees and in adults of bumble bees and solitary bees).
- Objective 2: test the chronic oral toxicity of multiple pesticides and contaminants in adult honeybees, bumble bees and solitary bees.
- Objective 3: feasibility study for the development of DEBtox models using acute and chronic toxicities of single and multiple pesticides and contaminants in bees (i.e. in adults and larvae of honeybees and in adults of bumble bees and solitary bees).

This contract was awarded by EFSA to: Centre for Ecology & Hydrology, the United Kingdom (UK)

Contractor: Centre for Ecology & Hydrology, MacLean Building, Benson Lane, Wallingford, Oxon, OX10 8BB, UK

Contract title: Chronic oral lethal and sub-lethal toxicities of different binary mixtures of pesticides and contaminants in bees (*Apis mellifera*, *Osmia bicornis* and *Bombus terrestris*).

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1.2. Additional information relevant to the specific design of exposures, including mixture experiments

One of the key uncertainties regarding possible field relevant ecotoxicological effects on bees is the response at an individual and population level to chemical mixtures. For most chemical mixtures in bees species, it is not established which, if any chemical mixtures operate in an additive manner according to the mode of action (i.e. similar, dissimilar) and which, if any show interactive joint effects (i.e. antagonistic, synergistic). Among interactive mixtures, those that show synergism are the ones that may provide the greatest concern as these have the possibility to result in joint effects in the field that would exceed those predicted based on information obtained from studies with the single chemical alone. Conducting assessment for interactive toxicity is feasible for some of the priority chemical mixtures that are likely to be present in key landscapes. These would include pesticide classes that are routinely jointly applied to flowering crops as well as pesticides and environmental contaminants that may co-occur as a result of agrochemical use and diffuse or point source pollution.

There are existing statistical tools and process based models available to investigate the effects of chemical mixtures. These include 'MIXTOX' model approaches that assess binary (Jonker et al., 2005) and ternary mixture datasets (Cedergreen et al., 2012) to test for synergistic, antagonistic, dose ratio and dose level dependent deviations in joint effects from the prediction of both concentration addition (CA) and independent action (IA). Known weaknesses of these statistical tools are that they 1) lack mechanistic basis for assessing chemical responses and the possible toxicokinetic and toxicodynamic changes that may underlie interactive toxicity and 2) that they provide only a snapshot of joint toxicity with time rather than a comprehensive assessment over the full time course of exposure and effect. Because of the underpinning mechanistic basis and their use of time course effect data, DEBtox models are suitable tools that can be used to analyse mixture data to provide information on the causes and consequence of joint effects and their interactions. DEBtox (Jager et al., 2007; Baas et al. 2010; Jager et al., 2011) models have been applied to the effects of chemical toxicants on survival

rate to establish no effect concentrations for a number of organisms in environmental risk assessment. DEBtox models for mixture effect on patterns of survival in time have been developed (Baas et al., 2007; Baas et al., 2009; Baas et al., 2010) and have been extended to include effects on multiple endpoints (Jager et al., 2010). The potential of these models to describe mixture effects for bees over extended exposure time (i.e. beyond 96 h) has not been explored until this project.

There are existing reports of the presence of synergism and antagonism for the joint effects of pesticides on different bee species. These include cases where large-magnitude synergisms have been observed for the interaction between tau-fluvalinate (a pyrethroid used as an acaricide) with sterol biosynthesis inhibiting fungicides (Johnson et al., 2013) and between tau-fluvalinate and the organophosphate comaphous also used for mite control (Mao et al., 2011). In both cases, interactions between the compounds at the active sites of the cytochrome P450 enzymes involved in first phase metabolism in bees was the underlying mechanism identified for the interaction. In other studies, smaller scale interactions causing synergism have been identified. These include interactions between neonicotinoids and sterol biosynthesis inhibiting fungicides (Thompson et al., 2014) of smaller (< 3 fold) maximum magnitude and those for a range of pesticides used in orchards with sterol biosynthesis inhibiting fungicides (Biddinger et al., 2013).

To extend the research capacity to investigate further field relevant mixtures there is a need to assess the application of existing mixture tools for analysis of data from toxicity tests conducted with bees. It is important to align toxicity testing approaches with the needs of the statistical and modelling tools applicable for data analysis. This will allow assessments of whether existing mixture models based on concepts such as concentration addition (CA) and independent action (IA) or derived from energy budget theory are able to describe the joint effects of non-interacting mixture and also to test hypotheses relating to the presence of synergistic or antagonistic interactions (the details of these concepts are discussed later in this report). A first step is to assess how such tools work in respect to studies of acute and chronic toxicity of chemicals (pesticides and contaminants) in bees. For such studies, comparative assessment with a range of bee species and life stages (adults and larvae) can provide comparative tests of suitability of current approaches to joint effect assessment of chemical mixtures including experimental set-ups, test designs, data analysis and modelling methods.

2. Data and Methodologies

2.1. Overall approach

Toxicity tests were conducted to assess the oral toxicity of seven chemicals and selected binary mixtures to three bee species; the eusocial bees *Apis mellifera* (honeybee) and *Bombus terrestris* (bumblebee) and a solitary bee *Osmia bicornis* (mason bee). These species were selected as being representative of bees with different social and ecological behaviour. A common testing method was developed for adults of all three species, based on previous published work, to assess acute and chronic oral toxicity of the study chemicals.

2.2. Experimental work plan

The work program consisted of four phases that each addressed specific issues in the assessment of the effects of oral chemical exposure on bee species:

- Phase 1 in this first phase, test hoarding containers were designed to ensure bees could be maintained for robust characterisation of chemical concentration effects on adult mortality for up to 10 days exposure for the three bee species. This extended previous work and protocols for acute and chronic exposures in terms of both the number of species and exposure times. In addition, methods were developed to conduct larval assays for *A. mellifera* only.

- Phase 2 used the method developed in Phase 1 to undertake dose response tests for seven test chemicals and compare their potencies in *A. mellifera* adults. DEBtox was used to estimate a time independent effect parameter (the "no effect concentration' or NEC) and toxicokinetic ('elimination rate') and toxicodynamic ('killing rate') values. From these data the effect of exposure times on LC_{50} values could be calculated (relevant to the time points within the test of 48 h, 96 h and 240 h). Appendix B contains the full description of the structure of the DEBtox models used.
- Phase 3 applied the Phase 2 single chemical testing approach to assess the comparative toxicity of the same seven chemicals for *B. terrestris* and *O. bicornis* using the experimental designs optimised in Phase 1. The LC_{50} values and parameter estimates from DEBtox allowed assessment of both the sensitivity of each species separately to the different tested chemicals and the relative sensitivity of the different species to each of the chemicals.
- Phase 4 used the single chemical toxicity results generated from Phases 2 and 3 to design two different types of test to assess the joint effects of binary chemical mixtures on the three bee species. The first tests were 'Potentiation' experiments in which only one of the two tested chemicals showed a significant concentration response in the Phase 2 and Phase 3 studies. The second tests were 'Toxicity' experiments in which the chemicals combined both showed a significant concentration response in Phase 2 and Phase 3 studies. Explicit details of these mixture designs are given in section 2.3.2.

2.3. Chemical selection and experimental design (Phase 2 & 3 tests)

2.3.1. Single chemicals

Seven chemicals were selected to reflect current concerns about agrochemicals and trace pollutants in the environment and also investigate different metabolic pathway targets. These were:

- CLOTHIANIDIN: a neonicotinoid insecticide which is widely used as a systemic insecticide and has a high potency to bees;
- TAU-FLUVALINATE: a pyrethroid insecticide which is widely used as an insecticide in pollinated crops and as a varroacide in hives, sohas a relatively low potency for bees;
- 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D): a synthetic auxin herbicide;
- PROPICONAZOLE: a conazole fungicide which is from a group of fungicides that have been reported as potential synergists;
- ARSENIC: the metalloid which known to be highly toxic and to effect the genome;
- CADMIUM: the non-essential heavy metal due to its known long-term effects;
- DIMETHOATE: an organophosphate insecticide, also used by the OECD as a reference toxicant in routine toxicity testing for honeybees and other arthropod species (OECD 1998)

Table 1: Selected chemicals for study for bee toxicity testing to derive effects concentrations for priority chemicals

	Current usage	Exposure scenario	Mechanism of action	Metabolism	Other information	Select
Neonicotinoid						
Chothianidin	Systemic seed treatment oilseed rape/beet	Nectar and pollen	Binds to nicotinic acetylcholine receptors causing overstimulation	Cytochrome P450, such as CYP6G1 in *D. Melanogaster* so P450 inhibition could give synergism	Clothianidin is first metabolite of Thiamethoxam.	As representative neonicotinoid
Pyrethroid						
Tau-fluvalinate	Spray used on oilseed rape. In hive varroacide	Contact in field and hive during feeding	Binds to voltage-gated sodium channels in to depolarise nerves	Metabolised by CYP9Q1, CYP9Q2, and CYP9Q3 in honeybees	Low affinity for bee sodium channel mean less toxic to bees than other pyrethroids	As representative pyrethroid
Organophosphate						
Dimethoate	Reference toxicant used for bee toxicity testing	Folia exposure and drinking water if used	Cholinesterase inhibition after metabolism to the oxon-metabolite	Metabolised by CYP3A$ in rat to oxon-metabolite	Typical organophosphate. Water solubility allows oral exposure.	A reference toxicant and organophosphate
Fungicide						
Propiconazole	Used widely as spray fungicide on oilseed rape	Foliar exposure during feeding on oilseed rape	Demethylation of C-14 in ergosterol biosynthesis, leading to accumulation of C-14 methyl sterols	Extensively metabolised in rat. Wide range of metabolites identified	Interacts with respiratory chain, so could affect energy metabolism	As representative fungicide
Herbicide						
2,4-dichlorophenoxyacetic acid,	common systemic herbicide used in the control of broadleaf weeds	Foliar exposure during feeding on oilseed rape	Synthetic auxin causing uncontrolled growth	Significant species differences in clearance in mammals	Potential effects on antioxidant systems	As representative herbicide
Metals & metalloids						
Cadmium	None but past industrial use	Soil contact	DNA damage, oxidative stress	Metallothionein	One of most toxic metals	As representative metal
Arsenic	None but past pesticide use	Soil contact (especially in arable areas)	DNA damage, Epigenetic effect on DNA methylation	Metallothionein and phytochelatins	Known toxicity	As representative metalloid

2.3.2. Mixture studies

Given that seven single compounds were tested in the single chemical testing phases 2 and 3 (as identified previously), there were 21 binary combinations that could have been tested as mixtures in Phase 4. Six combinations were selected based on a number of key considerations:

Suitability for potentiation or mixture toxicity designs

The single chemical tests (Phases 2 & 3) showed some chemicals produced a clear dose-mortality response, while for others no response was found, even up to maximum water soluble concentrations. This information was used to identify designs for Phase 4 mixture testing:

- For combinations where only one of the two tested chemicals showed a significant concentration response, the mixture effect could be assessed as a 'potentiation' change in sensitivity to the toxic chemical, as a result of the presence of the second chemical (that itself has no effect; see Figure 1A). For such designs, the concentration of the second 'potentiating' chemical was 10 x reported environmental concentrations to represent a plausible environmental worst case (EFSA PPR Panel, 2012). In some experiments, a further treatment series was used at 100 x the environmental concentrations for the potentiating chemical as a toxicological case study.
- Where both chemicals showed toxicity, a more classic mixture experimental design was used. This was based on a Concentration Addition (CA) concept; chemicals of similar mode of action contribute to a joint effect in amounts defined by the concentration and potency. This design includes effects at different levels and mixture ratios. Inclusion of single chemical treatments at the same levels as used in the mixture treatments allows the data to also be analysed against Independent Action (IA) model predictions in which chemicals contribute separately to the mixture effect independently in relation to any effect they have on their own (see Figure 1B).

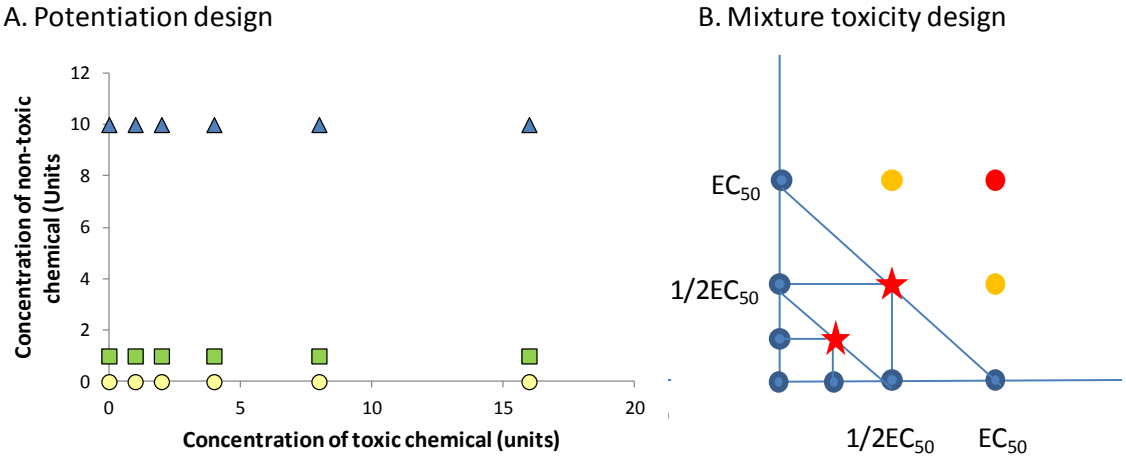


Figure 1: Designs for mixture experiments for cases where only one tested chemical shows a concentration response (A. Potentiation design) and where both chemicals show a concentration response (B. Mixture toxicity design)

Environmental realism

The chemical mixtures chosen for testing were selected to represent a range of possible mixture exposures (i.e. combinations to which bees could plausibly be exposed during their lifetime). Bees were orally exposed to chemicals through dosing the sucrose solution used as food. The use of

sucrose solutions for exposure presented some problems with chemicals with low water solubility. For some of the chemicals, other exposure routes may also be important in natural systems. For example direct contact could occur for some pesticide when used as sprays on some flowering crops. Contact with tau-fluvalinate may also occur when used as a varroacide in mite infected hives. Exposure through feeding, however, delivers continuous exposure to give a better assessment of effects in time, while maintaining the capacity to assess dose.

Metabolic and toxicological pathways

Test mixtures were chosen to represent a number of different mechanistic binary exposure scenarios. These included situations where bees were exposed to two chemicals with the same biological target (e.g. on nerve function for the insecticides), even if action was not mediated by the same molecular initiating event (e.g. acetylcholinesterase binding for dimethoate, nicotinic receptor binding for clothianidin, sodium channel binding for tau-fluvalinate). A sterol biosynthesis inhibiting chemical (propiconazole) was included specifically, as chemicals are known to be metabolised in bees by the cytochrome P450 system. Chemicals known to effect epigenetic regulation e.g. arsenic, and to suppress metabolic rate (and hence resources available for potential detoxification) e.g. cadmium, were also included. Mechanistic considerations such as these may provide a suitable framework for identifying further combination mixtures of chemicals for testing in the future.

Based on the above considerations, six mixtures were selected for initial study in the test system exposing groups of the honeybee *A. mellifera* (Phase 4) for which there were three potentiation mixture designs and two toxicity designs (see table 2 for summary).

Potentiation design combinations:

- Clothianidin and propiconazole to assess whether the presence of the sterol inhibiting conazole fungicide resulted in a change (increase or decrease) in the toxicity of the neonicotinoid clothianidin which is known to be metabolised by the cytochrome P450 system.
- Dimethoate and propiconazole to assess whether the presence of the sterol inhibiting conazole fungicide resulted in a change (increase or decrease) in the toxicity of an organophospahte dimethoate, that is metabolically activated from the less toxic parent form to a more toxic metabolite dimethoxon by the cytochrome P450 system.
- Clothianidin and tau-fluvalinate to assess whether the presence of the less toxic pyrethroid tau-fluvalinate, that is actively metabolised by the cytochrome P450 system to a less toxic form, changes the metabolism of the highly toxic neonicotinoid clothianidin with resultant effects (increase or decrease) on toxicity.

Mixture toxicity design combinations:

- Clothianidin and dimethoate to assess combined effects for two insecticides that target nerve function through different receptors and for which first phase metabolism has different effects on toxicity with increase through conversion to dimethoxon for dimethoate and reduction through conversion to multiple first phase metabolites for clothianidin.
- Clothianidin and cadmium to assess combined effects to insecticides that target nerve function and chemicals that effect metabolic resources to assess interaction between neural and metabolic toxicity.
- Cadmium and arsenic to assess interaction between chemicals with different modes of action including change in metabolic rate and modification of methylation status of the epigenome.

The studies with *B. terrestris* and *O. bicornis* provided the chance to assess whether patterns of joint additive and interactive effects that were identified in *A. mellifera* were repeated in other bee species (see table 2 for summary). Three mixtures were prioritised based on mechanistic considerations and also prior information concerning possible interactions for chemicals known to affect cytochrome P450

activity. As for *A. mellifera*, this included three potentiation designs and one toxicity mixture design as follows:

Potentiation design combinations:

- clothianidin and propiconazole,
- dimethoate and propiconazole (*O. bicornis* only),
- clothianidin and tau-fluvalinate (*B. terrestris* only)

Mixture toxicity design combinations*:*

- Clothianidin and dimethoate

Table 2: Summary of mixture tests conducted in Phase 4 of the project

Mixtures	Cloth-Propic	Cloth-Tau	Clot-Dimeth	Propic-Demth	Cd-As	Cloth-Cd
A. mellifera	✓	✓	✓	✓	✓	✓
O. bicornis	✓		✓	✓		
B. terrestris	✓	✓	✓			

2.4. Test methods used for each bee species

Many current concerns about the effects of chemical exposures on pollinators relate to the interactions with systemically applied neonicotinoid insecticides. For this the main exposure route is oral, via nectar, pollen and plant guttation water mixed with other chemicals. Indeed, the EFSA Scientific Opinion on the risk assessment of plant protection products on bees (EFSA PPR Panel, 2012) indicates that nectar foragers are potentially the most exposed category. Our tests were based on design aspects taken from established OECD protocols for oral (OECD, 1998) and oral acute single exposure for larvae (OECD, 2014). All our experiments on adult bees involved continuous exposure (over 240 h) to different concentrations of the test chemicals added to sucrose solution supplied as food (50% sucrose w/v for *B. terrestris* and 20% sucrose w/v for *O. bicornis*). Bees were able to feed *ad libitum* but were not supplied with pollen. Tests with *A. mellifera* larvae were adapted from an exposure protocol reported in a recent OECD released draft guidance document for repeated exposure for larvae (OECD, 2014) but adapted using methods from Genersch et al. (2005). Larval exposure was done via feeding spiked food for 24 h only and then monitored for a further 48 h, giving a test duration of 72 h.

2.4.1. *Apis mellifera* adults

Queenright colonies of *A. mellifera* were established from nucleus hives and maintained according to best available local bee keeping practice (see Appendix A for full details). Tests used even aged, adult worker honeybees collected from frames containing young brood. Four (single chemical tests) or three (mixture tests) replicate hives were used for each treatment. Bees were collected from hives by gently shaking or brushing them from the frames into holding containers. These holding containers were chilled individually in a -20°C for 30–45 seconds (Human et al., 2013). This cooling allowed individuals to be easily dispensed into a specifically designed hoarding test cage using entomological forceps (see Appendix A). Each test replicate comprised groups of 10 bees from a single hive. After bees were dispensed into the hoarding cage, a feeder unit containing the test chemicals or control sucrose (+/- acetone) was placed into the cage through a pre-bored hole. Test hoarding cages were maintained in a controlled environment room at 25ºC ± 2°C ~ 60% relative humidity (RH) in the dark for the 10 day duration of the experiment. Over this exposure, the mortality of bees was recorded at regular interval (3 times daily until 96 h, thereafter daily) and also behaviour score as normal and aberrant (erratic movement, shaking, lethargy, failure to respond to stimuli).

2.4.2. *Apis mellifera* larvae

The *A. mellifera* larval toxicity tests used an improved method from the procedure outlined in the Draft OECD test guidelines (OECD, 2012) that significantly increased control larval survival during experiments (Genersch et al., 2005). Larvae were maintained individually on a diet comprising 33% of a solution containing 9% fructose and 9% glucose and 66% of Royal Jelly (resulting food therefore contains 66% Royal Jelly : 3% Glucose : 3% Fructose). On Day 1, 1 day old larvae from frames from within the selected replicate hives were transferred, in groups of 10, to individual cells in 24 well tissue culture plates containing 300 µl freshly prepared clean and un-spiked diet. A grafting tool was used to lift individual larvae out of the brood frame and transfer them to diet, ensuring that the same orientation of the larva was maintained such that the spiracles were upright, thereby preventing suffocation and ensuring successful feeding. Each plate had 6 wells loaded only with sterile water to ensure humidity within the plate was maintained. All plates were warmed to 35°C prior to loading larvae and once loaded, were kept a 35°C in the dark in an incubator with no fan circulation and under high humidity (trays of water placed in the bottom of the incubator). After collection from frames, the larvae were held on this fresh diet for an initial period of 24 h to ensure any handling deaths were eliminated, prior to exposure. After 24 h, larvae were transferred to new plates containing either 300 µl diet spiked with chemicals at the required exposure concentration or un-spiked control diet, and incubated for a further 24 h. Concentrations required to achieve specific doses were based on an assumed consumption of 30 µl of the diet per individual larvae. After 24 h incubation on the spiked diet, larvae were checked and any mortality recorded (dead larvae were opaque, flattened and showed no movement or feeding when examined under a binocular microscope). Larvae were transferred at 24 h to un-spiked diet and further checked at 48 h and 72 h for mortality. At 72 h, surviving larvae were weighed to assess sub-lethal effects on weight gain and growth.

2.4.3. *Bombus terrestris* adults

There is no standardised OECD protocol for toxicity tests with *B. terrestris,* however, the use of queenless micro-colonies for bumblebees has been established and used for a range of studies including assessing the toxic effects of genetically modified (GM) crops (Mommaerts et al., 2011; Laycock et al., 2012; Laycock et al., 2014). The basic method has been described by Regali and Rasmont (1995) and Tasei et al. (2000) and is also specifically recommended by EFSA PPR Panel (2012).

Small colonies (20–35 workers) of the UK native *B. t. audax* were obtained from NV Biobest, Belgium. On receipt the colonies were switched from their supplied food and fed *ad libitum* on 50% sucrose solution and freeze dried pollen. For each test, bees were taken from a minimum of 4 colonies and a maximum of 10. On the day of the test, three young adult worker bees of a similar size were removed with long forceps from a colony and loaded into the same hoarding cages used for t *A. mellifera* tests to form a micro-colony. Each replicate micro-colony was randomly allocated to treatments with a minimum of 3 replicates for each exposure treatment, each from different source colonies. The sucrose solutions were supplied using the same basic feeder design as for the honeybees, with the aperture widened slightly to allow improved access. The exposed micro-colonies were maintained in a dedicated constant temperature facility at 25 ± 2°C, ~ 60% RH, in the dark.

2.4.4. *Osmia bicornis* adults

The solitary bee species selected for toxicity testing *O. bicornis* is native to the UK and is known to have a physiology and ecology that is typical of solitary bee species within the *Osmia* genus. The species is commercially available, from a very small number of suppliers, as field collected pupae that are obtained from placing 'trap' nests into the wider environment i.e. not reared in captivity. Challenges for the development of a robust assay for this species included; lack of standardised rearing conditions, lack of knowledge about previous exposure to environmental contaminants,

potential for high levels of parasitism and pathogen infections, difficulty in getting the species to feed under laboratory conditions (it is known that individuals readily enter torpor) and difficulty in differentiating between the sexes prior to adult emergence. Several of these factors are likely to contribute to higher variability in parameters like rates of control mortality, when compared to the more homogeneous test populations that can be obtained from honeybee hives that are used for standard toxicity testing.

To date there has been only limited use of *Osmia* in toxicity testing (Ladurner et al., 2003; Tesoriero et al., 2003; Ladurner et al., 2005; Konrad et al., 2008). These studies, and initial trials conducted in early 2014, provided the basis for the design of an appropriate testing protocol during the project. Overwintered *O. bicornis* pupae were obtained from a German stock population ('Dr. Schubert Plant Breeding', Germany). Pupae were stored at 4±1°C, 65±10% RH with no light for up to four months with no obvious effect on viable emergence. On warming, bees took 1–4 days to emerge, with this time decreasing the longer that pupae were maintained under cold conditions. Male pupae were generally smaller and weighed less than female pupae which allowed cohorts with approximately equal number of males and females to be selected and adults emerged for testing. Emergence success was approximately 80–85% for both test years.

The hoarding test cages used for all experiments were the same basic design used for *A. mellifera* and *B. terrestris* with a smaller feeder volume. For all experiments, male and females were maintained individually in cages. Ten replicates (5 males and 5 females) were used for each test. Test units were maintained in a controlled temperature glasshouse at 22 ± 2°C, ~ 60% RH, under natural photoperiod which was found to be preferable to an indoor constant temperature room under artificial light.

This approach was used in Phase 3 of the work plan to assess the toxicity of the seven single chemicals to adult bees that confirmed the suitability of key aspects of the test design and methods. However, control mortality over the extended exposure duration of 240 h (< 20–50% at 240 h) was greater when compared to the two other species. The test design was optimised by altering the initial rearing and selection of emerged bees, which reduced levels of control mortality (typically to < 20% at 240 h). The optimised design was used for the potentiation and mixture toxicity experiments.

2.4.5. Data collection and endpoints

Adult mortality was recorded three times daily during the first 96 hours of the exposure period to allow acute toxicity to be assessed. Chronic toxicity was assessed by extending the initial acute tests from 96 to 240 h; during this period survival was monitored at 24 hour intervals. This approach is in agreement with the recommendations from the EFSA Scientific Opinion on bees and the Tender requirement to minimise the number of bees tested. For a number of experiments (see results) bees were also scored for behavioural signs of overt toxicity at each time point. This discriminated between bees showing 'normal' and those showing aberrant behaviour which such as erratic movement, shaking and/or lethargy and failure to respond to stimuli. In addition, feeding syringes were weighed at 48 h, 96 h and 240 h to give an indication of feeding rates (after correction for evapotranspiration measured using cages containing only a feeding syringe).

2.5. Data analysis

2.5.1. Concentration, dose and dose/mg bee calculation

Since exposed bees in our assays were able to feed continuously, the only time invariant exposure parameters that can be used for comparison of sensitivity across different time points is the chemical concentrations in sucrose solution. For DEBtox analyses, survival data for all time points and all concentrations are used for a model run. Since received dose depends explicitly on exposure time, this means that estimates of effect related to exposure doses (i.e. the amount of the chemical consumed by each bee) cannot be reported for DEBtox analyses. In contrast, for probit analyses, estimates of effects associated with consumed doses can be made based on consumption rates per unit time (e.g. at 48 h, 96 h and 240 h time points). Where bees were housed in groups this estimate corresponds to mean intake rates, calculated for the individuals surviving at that time point i.e. mean consumption per individual based on the feeding rate of that group. As bees are continually feeding during the test, the actual dose received by an individual bee will increase over time. Hence it may be possible for LD_{50} values to increase with time while conversely, LC_{50} values will decrease. Individual body weight data for bees in the toxicity tests were used to correct the doses to dose/mg bee tissue to assess the extent to which bee body size affects apparent sensitivity when related to concentration and total unscaled dose alone.

2.5.2. Single chemical and potentiating experiments

All single chemical experiments and the concentration series of chemicals used in the potentiating experiments (i.e. those that demonstrated a dose response when tested alone) included a full concentration response series of six treatments with appropriate controls. Probit analysis was conducted for each concentration series based on the assumption that there was no contribution of the potentiating chemical to the toxicity of the toxic chemical. From the fitted probit models for each single chemical effect series, the LC_{50} values for the effects of the tested chemical for each species at each specific time-point being considered was estimated. The LC_{50} values for mortality at 48 h, 96 h and 240 h were derived. Additionally, the model can be used to derive estimates for lower effect concentrations for e.g. LC_{10} or LC_{25} values but estimate LC values close to zero are difficult to measure and the variability is considerable so the reliability of such estimates should be treated with extreme caution. However, the use of DEBtox parameters provides an alternative way to derive these low effect concentration estimates; the DEBtox based approach has the further advantage that it is more robust because the estimates of effect concentrations are made using parameters derived from the data from all time points.

In addition to measuring effects on survival, behavioural data was also analysed using probit analysis on the number of individuals from those surviving that showed sub-lethal, impaired behaviour. Data collected in potentiation mixture tests where there was a concentration series used for clothianidin alone was analysed for all three bee species. Analysis of these data included tracking behavioural effects in time and also estimation of effect concentrations ($EC_{50,\ behaviour}$) from behavioural data to compare between species and also to LC_{50} values. The $EC_{50,\ behaviour}$ values were estimated by fitting probit models to the binomial response variables used in assessments (non-impaired, impaired).

2.5.3. Mixture toxicity experiments

The binary mixture effect was modelled using a descriptive approach using both CA and IA as reference models. The procedure used for model fitting was the same as described for single compounds, but both the single compound and the mixture data were modelled concurrently (Jonker et al., 2005; Svendsen et al., 2010). For each fit, an F-test was performed to test whether the reference model alone was statistically significant and, thus, correlated better to the experimental data than the null-hypothesis of no relationship between single concentration and mixture effects. The

CA and IA fits for the whole response surface were also compared with CA and IA predictions based upon the best fitting single concentration response curves for each individual experiment. This ensured that the reference model provided a good statistical description of observed mixture toxicity.

To test for the presence of interactions, additional parameters for synergistic/antagonistic (*a*), concentration ratio-dependent (b_{DR}) and effect level-dependent (b_{EL}) deviations were added. The synergistic/antagonistic interaction term was initially added using a zero value for *a* and the model parameters were then adjusted iteratively with the Solver function of Microsoft Excel. The statistical significance of any improvement made to the model when compared to CA or IA model alone was then assessed using a Chi-squared (χ^2) test (Jonker et al., 2005). If a significant improvement in model fit was seen with the synergism/antagonism model, these parameters were then included as starting values for further fits that included firstly the dependent and then the effect-level dependent models. If the synergism/antagonism model was not a significant improvement over CA or IA alone, then starting values for the concentration-ratio dependent and effect-level dependent models were taken from the reference model and b_{DR} and b_{EL} given a start value of zero. The best model description of the data was therefore selected through this iterative process of model fitting. Full details for interpretation of parameter values obtained from the descriptive modelling are available in Jonker et al (2005), Martin et al (2009) and GomezEyles et al (2009) and in Appendix B.

2.5.4. Single chemical and mixture analysis using DEBtox

Data on mortality for each of the three species provided time series data that were suitable for modelling of the pattern of effects using the DEBtox model. The approach we chose is based on mechanistic model for survival compatible with the principles of DEB theory. This took the form of a scaled one-compartment model to describe uptake and elimination rates and a hazard model to describe survival. This model needs four time-independent parameters to describe the whole time course of the toxic effect:

- The Blank Killing Rate, which is a measure of the rate of background mortality in a population not subject to any chemical exposure (hr^{-1}).
- The No Effect Concentration (*NEC*), a time-independent toxicological threshold below which no effects occur even after life-long exposure, it is expressed as an environmental concentration in mmol/L.
- The killing rate (k_k), the toxic potency of the compound (once the NEC is exceeded) expressed in $(mmol/L)^{-1}\ d^{-1}$.
- The elimination rate (k_e), which describes when the equilibrium between internal and external concentration is set, expressed in d^{-1}.

For comparing chemical potencies, the NEC is particularly important, as it represents the concentration at which increased hazard (e.g. mortality) will be realised following long-term exposure. Whether these effects are observed depends on the modelled toxicokinetics relative to the period of interest or observation; when chemicals are predicted to slowly build up an internal concentration, the full hazard may not be realised in a short-term laboratory test or even life-time exposure. This is because it takes time to build up an internal concentration and therefore to exceed the internal *NEC*. Once the internal *NEC* is exceeded the survival probability of an individual starts to deviate from that of the controls. The killing rate determines how fast this process will go. With an infinitely high killing rate death is immediate once the *NEC* is exceeded but with a low killing rate it takes more time before the survival probability drops to zero, given enough time the survival probability will go to zero. However, for some compounds the combination of slow kinetics with a low killing rate implies that the survival probability does not go to zero during the entire lifetime of the organism. To assess how toxicity expressed as the LC_{50} changes with exposure time, it is possible to use the DEBtox parameter values to estimate the LC_{50} for different time point. For this assessment, we used the DEBtox parameters to calculated LC_{50}s for the 24 h, 48 h, 96 h and 240 h time points. Further, it is possible to

extend the assessment of sensitivity to time points beyond those used for the tests. For this we chose three additional time points for effect prediction. These were 480 h, a time twice the length of the test; 720 h, a time approximately equivalent to the lifetime of a summer worker honeybee; and 2160 h, which is a duration approximately equivalent to the over wintering life-time of a worker bee.

For the analysis of effects in mixtures, DEBtox was applied following the framework initially proposed by Baas et al. (2007). Within this approach, the effects of exposure to two compounds are simultaneously analysed. For the exposure to two compounds simultaneously, the effects are described by the toxicity parameters of the individual compounds, extended with an interaction parameter. If there is no interaction, then effects in the mixture will be described by the values of the NEC, elimination rate and killing rate which should be very similar (e.g. within 2 fold) of those in the single chemical tests. If an interaction is found, then an additional parameter included in the model will provide a significantly improved fit of the model to observed effects in time over the whole dataset.

3. Results

3.1. Test design and optimisation

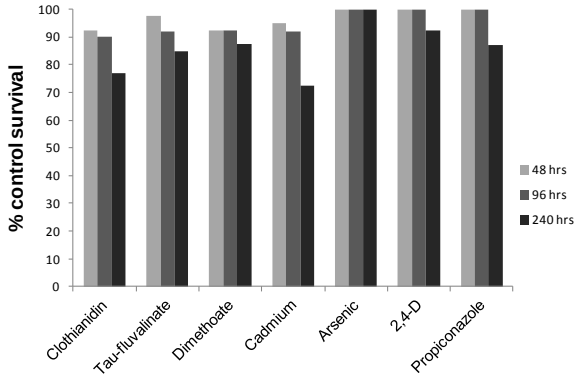
3.1.1. *Apis mellifera* and *Bombus terrestris* adults

The validation criteria for the OECD test for adult *A. mellifera* state that control survival rates should be greater than 90% of test individuals for tests lasting up to 48 h (OECD, 1998a) i.e. less than 10% control mortality. The extended chronic toxicity tests were run up to 240 h and were therefore optimised to ensure control survival rates were maintained as high as possible past the 48 h acute toxicity requirements for all species.

In the seven single chemical tests, control mortality rates for both *A. mellifera* and *B. terrestris* adults remained low up to 96h of testing with less than 10% control mortality for both species. Control mortality to the extended time point of 240 h for *A. mellifera* was greater than 10% in only 5 of the 7 single chemical tests, reaching a minimum of 22.5% in the cadmium tests. For *B. terrestris*, there was marginally increased control mortality at 240 h, reaching a maximum of 33% in the cadmium tests.

Mixture tests were done with bees from a different season but control mortality was still maintained at a low rate. At the 96 h test time point, this was less than 10% for *A. mellifera* in all tests and there was no control mortality in any test for *B. terrestris*. At the extended time point of 240 h, *A. mellifera* control mortality was less than 10% for all mixture tests except the arsenic/cadmium mixture where it was 15%. Similarly, control mortality for *B. terrestris* was below 20% for all mixture tests at the 240 h time point.

These acceptable low levels of background control mortality indicate that the bioassay test system developed was robust and ensured confidence that individual bees were not stressed during the course of the test.



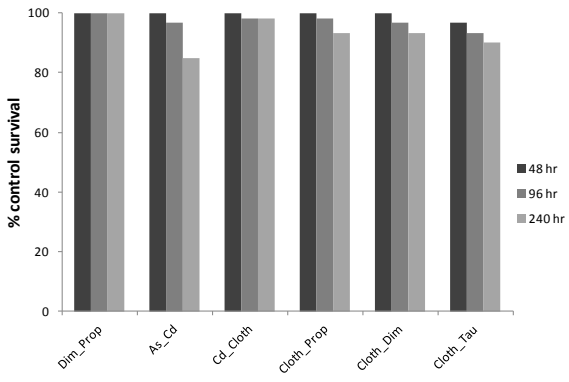


Figure 2: Control mortality after 48 h, 96 h and 240 h incubation of control bees in the toxicity tests with each of the selected single chemicals (a, left) and mixtures (b, right): all values are based on four replicates for single chemicals and three for mixture tests each containing 10 bees from a single hive

3.1.2. *Apis mellifera* larvae

The results of the six tests conducted with the single chemicals and the mixtures tests showed high survival of control larvae over the course of the test (Figure 3). At 48 h from initial exposure, control mortality was always below 20% and was below 10% in 4 of 6 cases. Extension of the incubation period to 72 h resulted in only marginally small increases in control mortality, and survival remained above 80% in 5 of 6 cases with the only exception being the dimethoate and propiconazole mixture experiment where it was 75% (Figure 3). As for the adult tests, this demonstrated a low level of stress and represent excellent bioassay conditions with low background mortality.

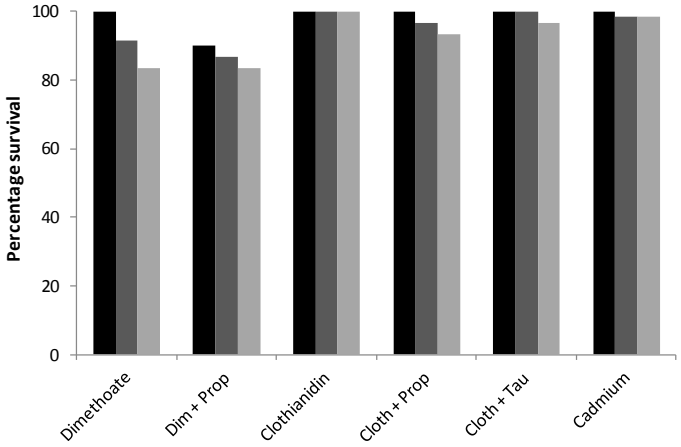


Figure 3: Control mortality after 24 h (black bar), 48 h (dark grey bar) and 72 h (light grey bar) incubation of *A. mellifera* larvae in the toxicity tests with each single chemicals and binary mixtures

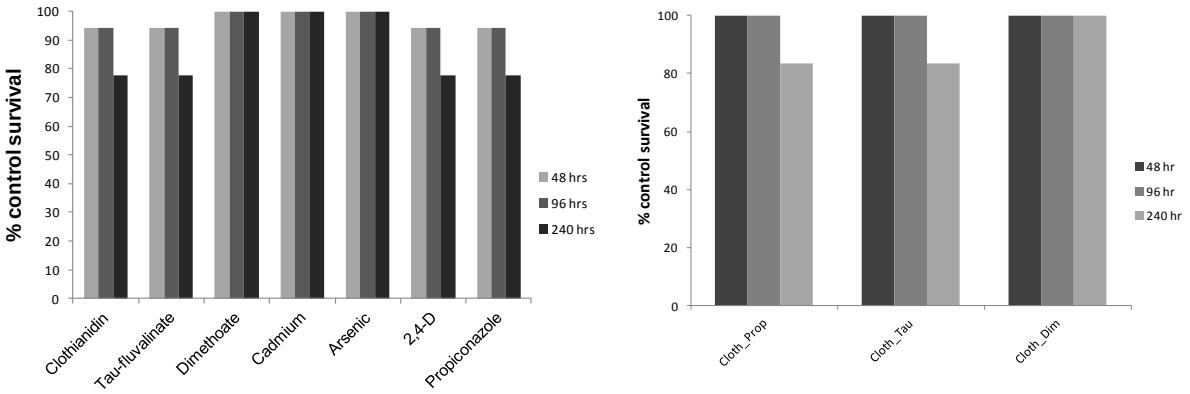


Figure 4: Control mortality after 48 h, 96 h and 240 h incubation of control bees in the toxicity tests with each of the selected single chemicals (left) and mixtures (right): all values are based on four replicates for single chemicals and three for mixture tests each containing 10 bees from a single hive

3.1.3. *Osmia bicornis* adults

Combined male and female control survival for adults included in the single chemical tests averaged 85% (range 70–100%) after 24 hr, being reduced to 75% (range 90–60%) and 67% (range 80–60% at 48 h and 96 h respectively and reduced further to 65% (range 80–40%) after 240 h. These temporal patterns of mortality indicated that the greatest losses occur in the first two days of the exposure. Thereafter extension of the exposure period was not associated with a greatly increased control mortality (Figure 5).

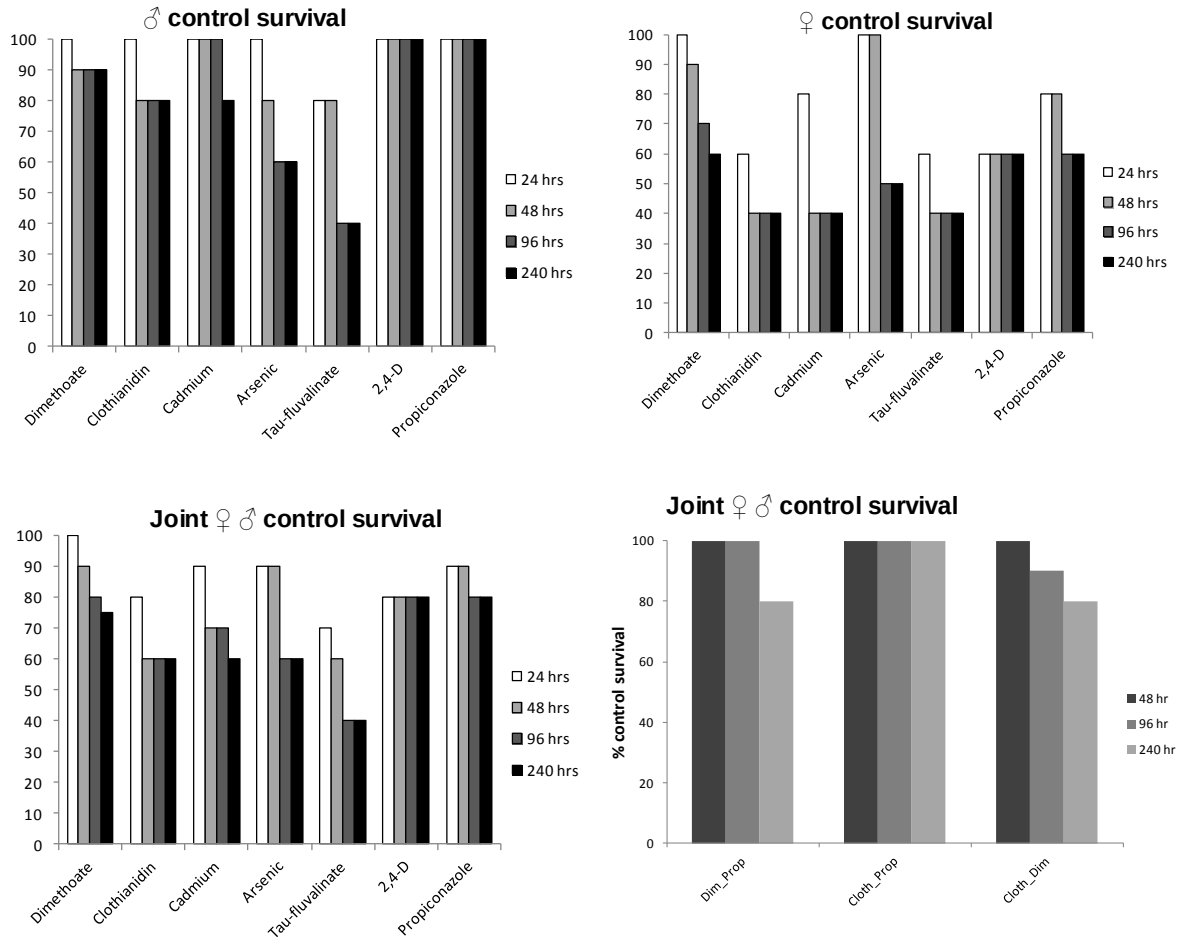


Figure 5: Survival of control treatment bees in the seven toxicity tests conducted with adult of *O. bicornis* showing percentage survival at each of four time points (24 h, 48 h, 96 h and 240 h) for males (top left), females (top right) and for the combined male/female populations (bottom left): all value for single sexes based on 5 individual except for dimethoate which is based on 10 data, combined data based on 10 individual except dimethoate which is 20 and survival in the three mixture tests at three time (48 h, 96 h and 240 h) for the combined male and female populations in the mixture test (bottom right)

This was a new test developed with this species and there could be a number of reasons for the relatively high initial rates of early control mortality found. The following potential reasons were investigated to determine if this contributed to control mortality:

- the extended period of time pupae were refrigerated prior to warming to encourage emergence.

- the use of co-solvent for dosing in tests conducted with three chemicals; tau-fluvalinate, propiconazole and 2,4-D;
- differences between sexes related to emergence time or initial feeding behaviour resulting from starvation post emergence;
- presence of a pathogen burden in the wild collected pupae.

Comparisons between experiments found no evidence of a temporal pattern in single chemical tests conducted later in the test season window compared to those conducted in the first weeks. There was also no difference in control mortality between tests with addition of solvent to control solutions, compared to non-solvent controls. To optimise the test design in the second season of testing in 2015, approaches to limit the impact of possible effects due to early starvation post emergence and the presence of pathogens were tested to see if a lower rate of background mortality could be achieved. A set of bioassay studies were conducted to track individual bee survival in experiments and to assess how different feeding strategies affected the survival of males and females over the 240 h test duration. The feeding regimes investigated were; initial post-emergence treatments of no feeding, feeding 20% sucrose solution or feeding 50% sucrose solution and then test maintenance diets of 20% sucrose solution or 50% sucrose solution.

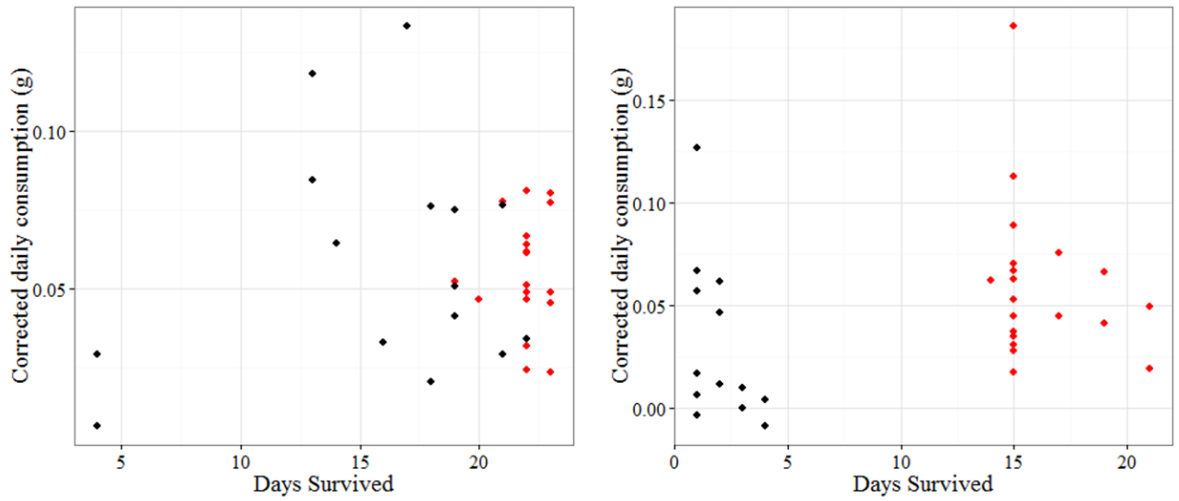


Figure 6: Survival of male (left) and female (right) *Osmia bicornis* after emergence in relation to daily consumption of sucrose solutions, highlighting a split in bee survival that indicates separation of a group of bees that fail to initially feed, and die early in the assays, and a group of bees that feed successfully upon emergence and survive for at least 10 days (the length of the toxicity bioassays)

The results of this observational study highlight two distinct patterns in the survival data (Figure 6). First there was a better survival of males than of females, with more males alive at the end of the 10 day maintenance period compared to females. Second, for both sexes but particularly for females, there was a clear split between individuals surviving for only a short period (< 5 days) and bees surviving beyond the 240 h period used for the toxicity tests. Initial feeding strategy (no feeding; feeding with 20% or 50% sucrose solutions) and changing the maintenance diet (20% or 50% sucrose solution) on survival over time failed to identify a feeding approach able to increase survival rates.

As neither initial food supply, nor the type of diet visibly improved control survival, it can be concluded from these feeding trials that neither of these factors caused the observed high control mortality in *O. bicornis*. Instead it appears that there are a proportion of females do not feed on emergence, although they stay alive for 2–3 days, that results in subsequent death. We currently have no information on what the nature of such a pathology may be but potentially could be related to asymptomatic pathogen infections. However, in a single chemical test with propiconazole conducted in 2014 (in a set of tests that had relatively high control mortality), we found that increasing exposure to this fungicide resulted in a recovery of survival from 50% in controls to near 100% survival in the highest exposed samples. Presence of an asymptomatic pathogen in emerged individuals may therefore warrant further study.

Based on the data from the initial survival trials we identified an improved approach to selection of adult *O. bicornis* for inclusion in toxicity tests. The approach followed that used in 2014 in which pupae were moved from storage at 4°C to an emergence temperature of 28°C. However, instead of individual incubation as used in 2014, pupae were instead transferred into flight cages in groups of 25 with 20% sucrose supplied as food. The majority of pupae emerged between 0–48 hours following warming and were then immediately placed individually in cages with 20% sucrose solution supplied for 3 days. During this time a number of bees died in accordance with the expectation from our initial trials and these deaths were primarily female bees with approximately 30% mortality noted. It was obvious after the 3 day period which bees had consumed sucrose as the levels in feeders were visibly reduced in those that had fed successfully. The remaining bees alive after this time were assumed to come from the cohort showing extended survival and were used for tests.

The improved design for the initial handling of emerged *O. bicornis* markedly increased control survival. There was a 100% control survival at 48 h, a > 90% survival at 96 h, and a > 80% survival after 240 h (Figure 6). Such increased rates of control survival provided a greatly improved basis for mixture (and single chemical) effect assessment.

3.2. Phase 2 - Toxicity of the seven test chemicals for the honeybee *Apis mellifera* including analysis using DEBtox

3.2.1. Experimental data and initial assessments of toxicity

The toxicity of the seven selected chemicals was assessed within intensively monitored extended (240 h) exposure assays. Two chemicals, 2,4-D and tau-fluvalinate, did not produce clear concentration or time dependent effects on survival relative to controls. For tau-fluvalinate, low mortality at intermediate concentrations, most notably at 10.7 and 26.8 µg/L, was observed. There was low mortality at the top concentration suggesting no clear concentration related effect on survival. This level of mortality was consistent with control mortality in some experiments. Propiconazole also did not produce a clear concentration dependent effect for time-points up to 144 h Thereafter, at the highest test concentration of 333 µg/L, increased mortality reached to 40% at 240 h. This effect was particularly pronounced for some colonies, with an LC_{50} close to the top tested concentration of 333 µg/L, with colony variations in sensitivity also seen.

The remaining four chemicals (dimethoate, clothianidin, cadmium and arsenic) all showed clear and consistent concentration and time dependent survival effects. All individuals exposed to the 5 highest dimethoate concentrations showed increased mortality. All bees exposed to the top three dimethoate concentrations were dead within 96 h. At 0.7 µg/L, the effect on mortality was not evident until after 96 h. LC_{50} (with 95% confidence intervals) were 2.2 (1.96–2.89) µg/bee at 48 h falling to the lowest value of 0.615 (0.459–0.77) µg/bee after 240 h of exposure. For clothianidin, bees exposed to concentrations of 0.037 mg/L and above showed clearly elevated mortality within 48 h. For clothianidin, LC_{50}s calculated by probit analysis decreased with time from 0.104 (0.072–0.137) mg/L

after 48 h to 0.055 (0.041–0.07) mg/L at 96 h and 0.016 (0.008–0.025) mg/L after 240 h (Table 3). For cadmium, mortality patterns were both strongly concentration and time dependent with consistent results over time and concentrations. Bees exposed to the highest concentration showed initial rapid mortality, followed sequentially by the remaining treatments to even the lowest tested concentrations. In a clear concentration dependent manner, LC_{50}s for cadmium were highly time dependent being 27.4 (0–64.1) µg/L at 48 h and ultimately 1.03 (0.37–1.64) µg/L at 240 h. Also, for bees exposed to arsenic, rapid mortalities were observed with all bees dead in the top two exposure concentrations after 96 h and in the third highest treatment after 148 h. Bees at intermediate concentrations also showed concentration and time dependent trends in mortality. For arsenic, LC_{50} were 25.7 (2.49–3.45) µg/L after 48 h and 4 (3.31–4.74) µg/l after 240 h.

3.2.2. Dynamic energy budget evaluation of survival effects including toxicokinetic and toxicodynamics

Two pesticides, tau-fluvalinate and the herbicide 2,4-D (Figure 7 a & b) did not show concentration related effects on survival. Consequently DEBtox models could not be fitted for these chemicals. For the remaining three pesticides, dimethoate, clothianidin and propiconazole (Figure 7c–e) and two trace metals (Figure 7f–g). DEBtox model could be fitted, both for the experimental population and also replicate colonies.

DEBtox fits suggest large differences in NEC values for the three pesticides (i.e. dimethoate, clothianidin and tau-fluvalinate), as well as differences in toxicokinetic and toxicodynamic traits that influence the estimated toxicity in time. The NEC for dimethoate of 0.41 µg/L was an order of magnitude higher than that for clothianidin indicating an intrinsic lower potency for the organophosphate. A slightly slower elimination rate is derived for dimethoate than clothianidin (0.4 vs 0.5 h^{-1}, respectively). Based on this value, internal concentrations take approximately 75 h to reach 95% of equilibrium. The comparatively high killing rate for dimethoate underpins strong time dependence for effects on survival (note that if the killing rate is infinitely high, death is immediate once the NEC is exceeded). This means that LC_{50} values approach the NEC within the test duration, being within a factor three and two of the NEC after the 96 h and 240 h and approximating to the NEC in a theoretical 720 h and 2160 h exposure (see Figure 7d. This analysis suggests that for dimethoate, toxicity data from short-term exposure tests can be used to derive estimates of toxicity for longer-term exposures.

The low NEC of 0.0533 mg/L for clothianidin reflects honeybee sensitivity to this chemical. This value is based on data with outlying values from one colony removed. This colony showed higher mortality at two intermediate exposure concentrations and the inclusion of these data lowers the NEC estimate, but obviously also gives a poorer model fit. The potential for colony effects and their effects on estimates of the NEC may warrant further investigation. Using elimination rate values derived from the restricted dataset, it is predicted that 95% of equilibrium body burden will be reached after approximately 60 h. The high killing rate based on this internal concentration defines a rapid progression of toxicity with time. Using DEBtox parameters to estimate the full time course of change in LC_{50} values over the exposure highlights how the LC_{50} rapidly approaches the NEC with extended exposure. For example, the 96 h LC_{50} is within a factor of 2 of the NEC and 240 h, 720 h and 2160 h LC_{50} approximates to the NEC. This time course of change in the LC_{50} indicates that for clothianidin, short-term test results provide a good approximation of the mortality effects that would occur during extended exposures (see Figure 7d).

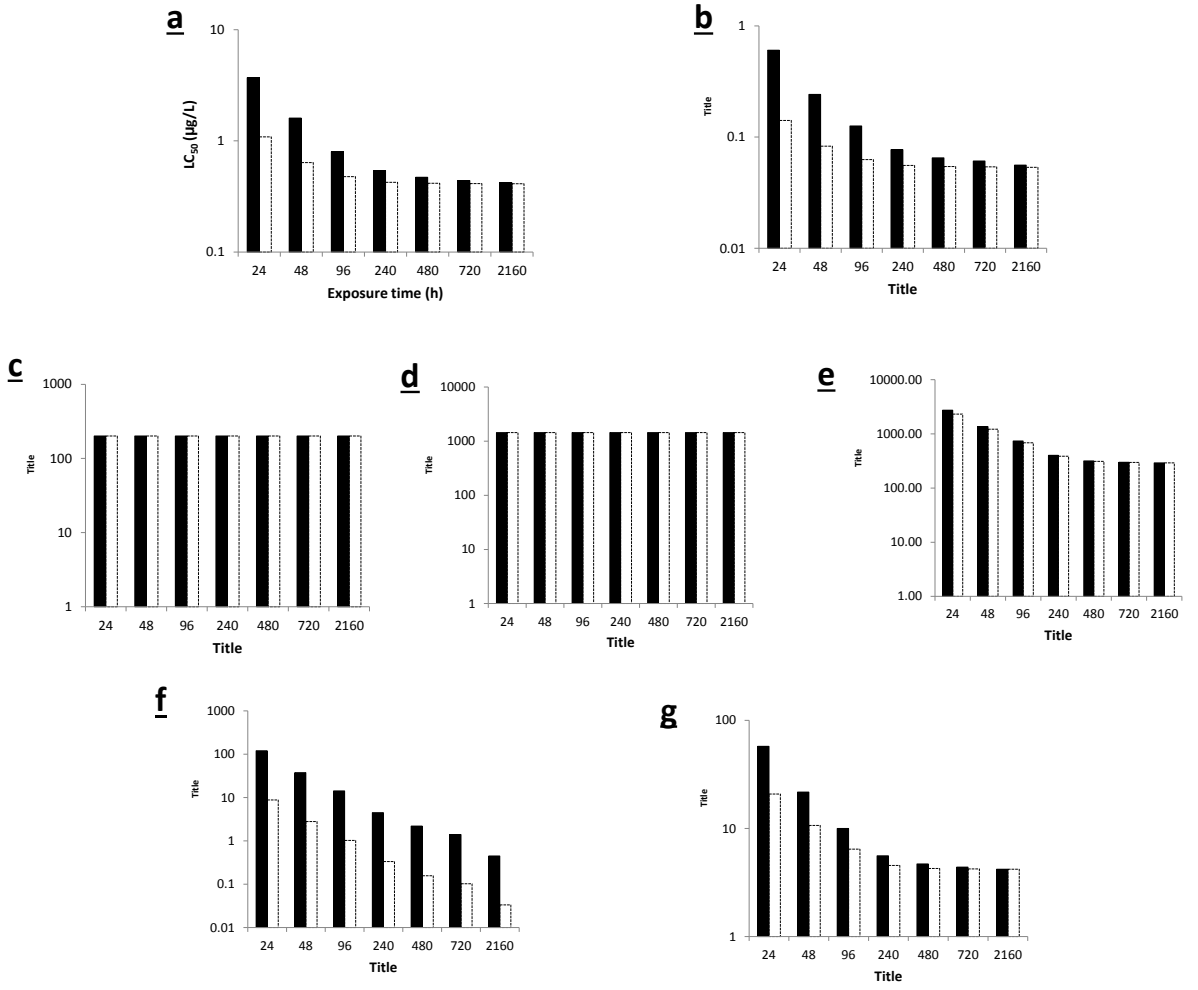


Figure 7: Relationship between LC_{50} values estimated from DEBtox model parameters for *Apis mellifera* exposed to a concentrations of (a) 2,4-D; (b) tau-fluvalinate; (c) dimethoate; (d) clothianidin; (e) propiconazole; (f) cadmium; (g) arsenic and exposure time estimated for time periods (24 h, 48 h, 96 h, 240 h) relating to the exposure and extended to periods (480 h, 720 h, 2160 h) relevant to the life-span of a worker bee during normal summer season and when overwintering

The fungicide propiconazole also showed an effect on survival in three of the four colonies, but only at the highest tested concentration. Here, the average of the three colonies showing an effect was taken to derive parameter values for this compound within the DEBtox model. The derived propiconazole NEC (292 µg/L) exceeds these for the two insecticides dimethoate and clothianidin by 4 and 5 orders of magnitude respectively. This is a relatively low rate of elimination, which suggests that bees will take 166 h to reach 95% of internal equilibrium. A lower killing rate results in a slow progression of toxicity in time in the treatments where the NEC is exceeded. After 240 h exposure, there is less than 50% mortality at the top exposure dose of 1000 µg/L which is just above the NEC. LC_{50}s estimated for 480 h, 720 h and 2160 h exposure relate closely to the NEC, so that for this chemical short-term test results provide a close approximation of long-term toxicity (Figure 7e).

Datasets for both trace metals resulted in reliable model fits (Figure 7 f–g). The NEC for cadmium was equivalent to zero which suggests that cadmium exposure at any concentration would result in higher mortality compared to unexposed bees in the experiment. The elimination rate of 0.037 h^{-1} corresponds to a time to 95% internal equilibrium of 80 h. Even though accumulation progresses to

equilibrium well within the exposure period, mortality rates increase relatively slowly due to the low killing rate. Hazard is strongly time dependent. LC_{50} reduces by a factor of > 8 (37.5 – 4.5 µg/L) when exposure increases from 48 h to 240 h. Further reductions of the LC_{50}s to 1.4 µg/L are predicted for 720 h exposure (and 0.45 µg/L after 2160 h exposure) (Figure 8f). This corresponds to a > 25 fold reduction in LC_{50} when the exposure period is extended from a 48 h laboratory test duration to a full adult worker life-span.

The mean arsenic NEC estimated using data for all four hives was 4.2 µg/L. However, there was a difference between colonies (1.74 µg/L to 5.6 µg/L) suggesting a three-fold difference in colony sensitivity. The mean elimination rate predicts a time to 95% internal equilibrium of 200 h. The killing rate for arsenic is relatively low, so although internal equilibrium is reached during the exposure period, toxicity progresses comparatively slowly. Thus, the LC_{50} at 96 h remains more than 10 fold above the NEC. When predictions are made for a 720 h exposure, the DEBtox estimate LC_{50} approaches the NEC, indicating that bees exposed for the full worker life-span will experience effects at close to the incipient LC_{50} (Figure 7g).

3.3. Phase 3 – Comparative toxicity of seven chemicals to *Apis mellifera, Bombus terrestris* and *Osmia bicornis*

3.3.1. Concentration, dose, and dose /mg bee as exposure metrics and different effect level estimates

The general pattern across chemicals of decreasing estimates of LC_{50} with increasing exposure times found for *A. mellifera* (Figure 8) were also found for the other species tested. When toxicity is expressed in relation to the received dose or as a weight corrected, dose / mg bee, to assess the extent to which bee body size affects apparent sensitivity, the main patterns is that dose conversion reduced the variation between parameter estimated made for different time points (see Tables 3–6).

For some chemicals, LD_{50}s show patterns of both decrease and increase over time depending on the species. For example clothianidin LD_{50}s decline in time for *A. mellifera*, but increase for *O. bicornis*; whereas Cd LD_{50}s are constant for *B. terrestris*, but decrease for *A. mellifera* and *O. bicornis*).

Table 3: Toxicity of seven chemicals to *A. mellifera* expressed as LC_{50} (with 95% CIs) calculated using probit analysis based on exposure concentration in food (mg/L), average consumed dose (mg/bee) and average dose / mg bee (mg/mg bee) for three time-points (48 h, 96 h, 240 h)

	48 h		96 h		240 h	
	Value	95% CIs	Value	95% CIs	Value	95% CIs
LC_{50} (mg/L)						
Dimethoate	2.42	1.96 - 2.89	1.16	0.95 - 1.38	0.62	0.46 - 0.77
Clothianidin	0.104	0.072 - 0.137	0.055	0.04 - 0.07	0.0167	0.01 - 0.02
Tau-fluvalinate	NC		NC		NC	
Propiconazle	NC		NC		NC	
2,4-D	NC		NC		NC	
Cadmium	18.4	9.1 - 27.6	3.69	0 - 11.91	NC	
Arsenic	25.7	22.2 - 29.1	13.6	12 - 15.1	4.03	3.31 - 4.74
LD_{50} (mg/bee)						
Dimethoate	0.339	0.27 - 0.4	0.32	0.3 - 0.39	0.43	0.322 - 0.539
Clothianidin	0.0146	0.010 - 0.019	0.0154	0.011 - 0.02	0.0117	0.0056 - 0.0168
Tau-fluvalinate	NC		NC		NC	
Propiconazle	NC		NC		NC	
2,4-D	NC		NC		NC	
Cadmium	2.58	1.27 - 3.86	1.03	0 - 3.33	NC	
Arsenic	3.60	3.11 - 4.07	3.81	3.36 - 4.23	2.82	2.317 - 3.318
LD_{50} by body weight (mg / mg bee)						
Dimethoate	0.00424	0.003 - 0.005	0.00406	0.003 - 0.005	0.00543	0.00403 - 0.0067
Clothianidin	1.82E-04	1.26E-04 - 2.40E-04	1.93E-04	1.40E-04 - 2.45E-04	1.46E-04	7.00E-05 - 2.10E-04
Tau-fluvalinate	NC		NC		NC	
Propiconazle	NC		NC		NC	
2,4-D	NC		NC		NC	
Cadmium	0.0322	0.016 - 0.048	0.0129	0 - 0.042	NC	
Arsenic	0.044975	0.039 - 0.051	0.0476	0.042 - 0.05	0.0353	0.029 - 0.0415

Table 4: Toxicity of seven chemicals to *B. terrestris* expressed as the LC_{50} (with 95% CIs) calculated using probit analysis based on exposure concentration in food (mg/L), average consumed dose (mg/bee) and average dose / mg bee (mg/mg bee) for three time-points (48 h, 96 h, 240 h)

	48 h		96 h		240 h	
	Value	95% CIs	Value	95% CIs	Value	95% CIs
LC_{50} (mg/L)						
Dimethoate	2.19		1.43	1.06 - 1.79	0.363	0.284 - 0.473
Clothianidin	0.038	0.021 - 0.053	0.025	0.018 - 0.033	0.0164	0.0104 - 0.0226
Tau-fluvalinate	44.7		55.34	33.2 - 77.5	61.96	7.22 - 11.6
Propiconazle						
2,4-D						
Cadmium	22.5	16.3 - 28.70	9.7	7.10 - 12.70	5.50	3.5 - 7.5
Arsenic	21.2	0 - 792.00	8.7	5.6 - 11.80	4.40	3 - 5.9
LD_{50} (mg/bee)						
Dimethoate	1.53		2	1.48 - 2.51	1.27	0.994 - 1.66
Clothianidin	0.0266	0.015 - 0.037	0.035	0.025 - 0.046	0.0574	0.0364 - 0.0791
Tau-fluvalinate	31.3		38.7	23.2 - 54.3	43.4	5.1 - 8.1
Propiconazle	NC		NC		NC	
2,4-D	NC		NC		NC	
Cadmium	15.75	11.4 - 20.1	13.6	9.9 - 17.8	19.25	12.3 - 26.3
Arsenic	14.84	0 - 554	12.2	7.8 - 16.5	15.40	10.5 - 20.7
LD_{50} by body weight (mg / mg bee)						
Dimethoate	0.00958		0.0125	0.009 - 0.016	0.00794	0.00621 - 0.0103
Clothianidin	1.66E-04	9.19E-05 - 2.32E-04	2.19E-04	1.58E-04 - 2.89E-04	3.59E-04	2.28E-04 - 4.94E-04
Tau-fluvalinate	0.196		0.242	0.145 - 0.339	0.271	0.0316 - 0.0508
Propiconazle	NC		NC		NC	
2,4-D	NC		NC		NC	
Cadmium	0.098	0.071 - 0.126	0.085	0.062 - 0.111	0.12	0.077 - 0.164
Arsenic	0.093	0 - 3.465	0.076	0.049 - 0.103	0.096	0.066 - 0.129

Table 5: Toxicity of seven chemicals to *O. bicornis* expressed as the LC_{50} (with 95% CIs) calculated using probit analysis based on exposure concentration in food (mg/L), average consumed dose (mg/bee) and average dose / mg bee (mg/mg bee) for three time-points (48 h, 96 h, 240 h)

	48 h		96 h		240 h	
	Value	95% CIs	Value	95% CIs	Value	95% CIs
LC_{50} (mg/L)						
Dimethoate	7.73	5.67 - 9.79	3.68	2.60 - 4.76	NC	
Clothianidin	0.042	0.014 - 0.069	0.031	0.009 - 0.053	0.028	0.006 - 0.051
Tau-fluvalinate	36	2.3 - 69.8	1	0.0 - 30.3	NC	
Propiconazle	NC		NC		NC	
2,4-D	NC		NC		NC	
Cadmium	27.4	0 - 64.1	2.2	0 - 7	1	0.4 - 1.6
Arsenic	50.5	0 - 105	3.1	0.0 - 7	NC	
LD_{50} (mg/bee)						
Dimethoate	1.546	1.13 - 1.96	1.47	1.04 - 1.90	NC	
Clothianidin	0.0084	0.003 - 0.014	0.0124	0.004 - 0.021	0.0280	0.00600 - 0.05100
Tau-fluvalinate	NC		NC		NC	
Propiconazle	NC		NC		NC	
2,4-D	NC		NC		NC	
Cadmium	5.48	0 - 12.82	0.88	0 - 2.80	1.00	0.40 - 1.6
Arsenic	10.10	0 - 21.0	1.24	0 - 2.80	NC	
LD_{50} by body weight (mg / mg bee)						
Dimethoate	0.0257667	0.019 - 0.03	0.0245333	0.017 - 0.03	NC	
Clothianidin	1.40E-04	4.67E-05 - 2.30E-04	2.07E-04	6.00E-05 - 3.53E-04	4.67E-04	1.00E-04 - 8.50E-04
Tau-fluvalinate	NC		NC		NC	
Propiconazle	NC		NC		NC	
2,4-D	NC		NC		NC	
Cadmium	0.0913333	0 - 0.214	0.0146667	0 - 0.047	0.0166667	0.007 - 0.027
Arsenic	0.1683333	0 - 0.350	0.0206667	0 - 0.047	NC	

Table 6: Toxicity of seven chemicals to *O. bicornis* expressed as the LC_{50} calculated from DEBtox model parameters using exposure concentration in food (µg/L), average consumed dose (µg/bee) and average dose / mg bee (µg/mg bee) for three exposure time-points (48 h, 96 h, 240 h)

	Apis mellifera				*Bombus terrestris*				*Osmia bicornis*			
	48 h	**96 h**	**240 h**	**720 h**	**48 h**	**96 h**	**240 h**	**720 h**	**48 h**	**96 h**	**240 h**	**720 h**
LC_{50} (mg/L)												
Dimethoate	1.6	0.8	0.54	0.44	1.7	0.61	0.21	0.12	11.5	4.8	2	0.98
Clothianidin	0.242	0.126	0.065	0.061	0.154	0.07	0.03	0.016	0.304	0.183	0.12	0.105
Tau-fluvalinate	*201*	*201*	*201*	*201*	*104*	*59*	*35*	*28*	*930*	*466*	*189*	*67*
Propiconazle	335	300	292	291	351	289	270	266	1000	1000	1000	1000
2,4-D	1438	1438	1438	1438	900	900	900	900	2850	2850	2850	2850
Cadmium	37.5	14.2	4.5	1.4	22	8.1	3.1	1.6	56	14	2.2	0.26
Arsenic	21.7	10	5.6	4.4	19	11	7.6	7	57.3	22.4	8.3	3.5
LD_{50} (mg/bee)												
Dimethoate	0.224	0.224	0.378	0.924	1.19	0.854	0.735	1.26	2.3	1.92	2	2.94
Clothianidin	0.03388	0.03528	0.0455	0.1281	0.1078	0.098	0.105	0.168	0.0608	0.0732	0.12	0.315
Tau-fluvalinate	*28.14*	*56.28*	*140.7*	*422.1*	*72.8*	*82.6*	*122.5*	*294*	*186*	*186.4*	*189*	*201*
Propiconazle	*46.9*	*84*	*204.4*	*611.1*	*245.7*	*404.6*	*945*	*2793*	*200*	*400*	*1000*	*3000*
2,4-D	201.32	402.64	1006.6	3019.8	630	1260	3150	9450	570	1140	2850	8550
Cadmium	5.25	3.976	3.15	2.94	15.4	11.34	10.85	16.8	11.2	5.6	2.2	0.78
Arsenic	3.038	2.8	3.92	9.24	13.3	15.4	26.6	73.5	11.46	8.96	8.3	10.5
LD_{50} by body weight (mg / mg bee)												
Dimethoate	0.0028	0.0028	0.00473	0.01155	0.00744	0.00534	0.00459	0.00788	0.03833	0.0320	0.03333	0.049
Clothianidin	4.24E-04	4.41E-04	5.69E-04	1.60E-03	6.74E-04	6.13E-04	6.56E-04	1.05E-03	1.01E-03	1.22E-03	2.00E-03	5.25E-03
Tau-fluvalinate	*0.352*	*0.704*	*1.759*	*5.276*	*0.455*	*0.516*	*0.766*	*1.838*	*3.1*	*3.107*	*3.150*	*3.35*
Propiconazle	*0.586*	*1.05*	*2.555*	*7.639*	*1.536*	*2.529*	*5.906*	*17.456*	*3.333*	*6.667*	*16.667*	*50*
2,4-D	*2.5*	*5*	*12.6*	*37.7*	*3.9*	*7.9*	*19.7*	*59.1*	*9.5*	*19*	*47.5*	*142.5*
Cadmium	0.0656	0.0497	0.0394	0.0368	0.0963	0.0709	0.0678	0.105	0.1867	0.0933	0.0367	0.013
Arsenic	0.038	0.035	0.049	0.1155	0.0831	0.0963	0.1663	0.4594	0.191	0.1493	0.1383	0.175

In addition to estimating LC/LD_{50} values from the time series survival data, it is also possible to estimate lower effect level concentrations and dose. The relationships between these low effect level concentrations (e.g. LC_5), the LC_{50} and the NEC are themselves highly time dependent. The relative values of these parameters in time are dependent on the toxicokinetics and toxicodynamics of each chemical.

For substances that reach an internal equilibrium in the tests and that have a relatively high killing rate, there will be a relatively rapid progress of toxicity; all individuals in treatments exposed above the NEC will exceed the internal threshold concentration for effects and this exceedance will result in overt toxicity and mortality. The result of this is that over time the LC_{50} value will approach the NEC value. At this point, the slope of the concentration response curve will be very steep and values such as the LC_5 and LC_{50} will be effectively equivalent. When this is the case, there is no benefit from estimating low effect level values in addition to the LC_{50}.

For chemicals with slow toxicokinetics, there will be a slow progression of effect, driven by the fact that internal concentrations take extended time to reach internal equilibrium. When elimination rate is close to zero, predictions can be made using time spans longer than the actual lifetime of the species. This is the case in this study for cadmium in *A. mellifera*. Only in such cases do LC_5 estimates contribute additional information to the assessment. A full table of derived LC_{50} and LC_5 values from the DEBtox model fits that allow comparison also for the NEC is presented in Table 7 A–C for values calculated according to exposure concentrations, received dose and dose by bodyweight respectively.

Table 7: DEBtox calculated effect concentrations for the five of seven chemicals showing overt toxicity for effects on survival in *A. mellifera*, *B. terrestris* and *O. bicornis* identifying how toxicity values changes over time to converge with time to approach the DEBtox NEC for the majority of chemicals in the 240 h exposure for concentrations and is affected by the continuous consumption for the dose related metrics

A) LC_5 and LC_{50} values

	48 h LC_5	96 h LC_5	240 h LC_5	720 h LC_5	48 h LC_{50}	96 h LC_{50}	240 h LC_{50}	720 h LC_{50}
Apis mellifera								
Dimethoate	0.64	0.48	0.42	0.41	3.78	1.55	0.54	0.45
Chlothianidin	0.083	0.063	0.056	0.054	0.671	0.248	0.078	0.061
propiconazole	1217	685	388	296.8	2731	1363	403	299
Arsenic	10.7	6.44	4.56	4.23	57.5	22.0	5.65	4.48
Cadmium	2.79	1.02	0.33	0.1	118	37.68	4.52	1.39
Bombus terrestris								
Dimethoate	0.52	0.25	0.12	0.08	1.73	0.61	0.21	0.10
Chlothianidin	0.102	0.053	0.025	0.015	0.151	0.069	0.030	0.016
Arsenic	14.7	9.52	7.22	6.92	18.7	11	7.61	6.98
Cadmium	3.50	1.87	1.22	1.05	22.1	8.26	3.18	1.63
Osmia bicornis								
Dimethoate	1.49	0.88	0.61	0.52	11.7	4.99	2.07	0.98
Chlothianidin	0.306	0.185	0.121	0.107	0.308	0.186	0.121	0.107
Arsenic	6.12	3.06	1.81	1.38	58.3	23.0	8.57	3.49
Cadmium	3.82	0.96	0.15	0.02	51.6	12.9	2.07	0.23

B) LD_5 and LD_{50} values

	48 h LC_5	96 h LC_5	240 h LC_5	720 h LC_5	48 h LC_{50}	96 h LC_{50}	240 h LC_{50}	720 h LC_{50}
Apis mellifera								
Dimethoate	0.09	0.13	0.30	0.87	0.53	0.43	0.38	0.94
Chlothianidin	0.01	0.02	0.04	0.11	0.09	0.07	0.05	0.13
propiconazole	170	192	272	623	382	382	282	629
Arsenic	1.49	1.80	3.19	8.88	8.05	6.17	3.96	9.41
Cadmium	0.39	0.29	0.23	0.22	16.52	10.55	3.16	2.92
Bombus terrestris								
Dimethoate	0.37	0.35	0.43	0.88	1.21	0.86	0.73	1.07
Chlothianidin	0.07	0.07	0.09	0.16	0.11	0.10	0.10	0.17
Arsenic	10.28	13.33	25.26	72.64	13.06	15.40	26.64	73.28
Cadmium	2.45	2.61	4.27	11.06	15.47	11.56	11.13	17.14
Osmia bicornis								
Dimethoate	0.30	0.35	0.61	1.56	2.34	2.00	2.07	2.95
Chlothianidin	0.06	0.07	0.12	0.32	0.06	0.07	0.12	0.32
Arsenic	1.22	1.23	1.81	4.14	11.65	9.18	8.57	10.47
Cadmium	0.76	0.38	0.15	0.05	10.32	5.16	2.07	0.69

C) LD_5 and LD_{50} values by body weight

	48 h LC_5	96 h LC_5	240 h LC_5	720 h LC_5	48 h LC_{50}	96 h LC_{50}	240 h LC_{50}	720 h LC_{50}
Apis mellifera								
Dimethoate	0.0011	0.0017	0.0037	0.0108	0.0066	0.0054	0.0047	0.0117
Chlothianidin	1.45E-04	2.19E-04	4.87E-04	1.41E-03	1.17E-03	8.67E-04	6.86E-04	1.60E-03
propiconazole	2.13	2.40	3.39	7.79	4.78	4.77	3.52	7.86
Arsenic	0.019	0.023	0.040	0.111	0.101	0.077	0.049	0.118
Cadmium	0.005	0.004	0.003	0.003	0.207	0.132	0.040	0.036
Bombus terrestris								
Dimethoate	0.0023	0.0022	0.0027	0.0055	0.0075	0.0053	0.0045	0.0067
Chlothianidin	4.45E-04	4.60E-04	5.56E-04	1.00E-03	6.61E-04	6.05E-04	6.48E-04	1.06E-03
Arsenic	0.0642	0.0833	0.1579	0.4540	0.0817	0.0962	0.1665	0.4580
Cadmium	0.0153	0.0163	0.0267	0.0691	0.0967	0.0723	0.0695	0.1071
Osmia bicornis								
Dimethoate	0.0050	0.0058	0.0102	0.0260	0.0390	0.0333	0.0345	0.0491
Chlothianidin	1.02E-03	1.24E-03	2.02E-03	5.36E-03	1.03E-03	1.24E-03	2.02E-03	5.36E-03
Arsenic	0.0204	0.0204	0.0301	0.0690	0.1942	0.1531	0.1428	0.1745
Cadmium	0.0127	0.0064	0.0026	0.0009	0.1720	0.0860	0.0344	0.0115

3.3.2. Comparative toxicity of different chemicals

Exposure to the chemicals resulted in clear dose related effects on patterns of survival in time for some, but not all, of the insecticides, other pesticides and trace elements tested. Comparisons with previous results indicate this is in general agreement with published data where that is available (e.g. Bertholf and Pilson 1941; Cronn 1991; Iwasa et al., 2004; U.S. EPA Environmental Protection Agency 2005; Johnson et al., 2013; Laurino et al., 2013). For the three tested insecticides, toxicity was in the order clothianidin > dimethoate >> tau-fluvalinate in all species. The difference in toxicity between the most toxic insecticide clothianidin, and least toxic insecticide tau-fluvalinate, was approximately a factor of 1000 in *B. terrestris* and *O. bicornis* and a factor of over 20,000 for *A. mellifera*. The high affinity of clothianidin and dimethoate for receptors within the insect nervous systems are key to defining their relatively high potency. The low toxicity of tau-fuvaliante can be linked to the extensive metabolism of this compound by cytochrome P450 as shown in the honeybee (Johnson et al., 2006).

The herbicide 2,4-D and fungicide propiconazole showed only minimal effects on survival in time for *O. bicornis* and *A. mellifera*. There was no effect of 2,4-D on any tested species at exposure concentrations up to maximum water solubility. The low toxicity of this compound suggests the absence of a specific relevant mechanism of action in bee species. Propiconazole showed toxicity only at high concentration treatment for *A. mellifera* and *B. terrestris*. Sterol biosynthesis inhibiting fungicides have been linked to inhibition of cytochrome P450 14-alpha-demethylase which is involved in natural and xenobiotic toxicants. This gives the potential for more subtle effects on metabolic and hormone disruption that may be revealed only at the colony level. Both trace metals showed a similar strong concentration (dose) and time dependent toxicity as found for *A. mellifera*. The time dependence of toxicity was strongest for cadmium in all three species. This likely reflects the slow elimination rate of this metals resulting in an increasing effect with exposure time.

3.3.3. Comparative sensitivity of different species

Comparisons of toxicity metrics calculated by time point probit analysis or from DEBtox parameters between species concentration, dose, and dose / mg bee can be used to express the range of species sensitivity (Table 8). Calculation of species:species ratio for different metrics show only a relatively small range of species sensitivity. Approximately 50% of all values whether expressed as concentrations or as dose, were within a factor of 2 between the species. All other values, with the exception of two, are within an order of magnitude indicating that in the large majority of cases variation in species sensitivity fell within a range of a factor of 10. Larger variations in sensitivity were found for cadmium calculated as point estimate LC_{50} values for *O. bicornis* and *B. terrestris.* The difference in calculated values range up to 15 fold for this metal between these two species. Notably, the largest difference in toxicity between these two species always occur when the effect is calculated using consumed dose as the exposure metric. When exposure is expressed as concentrations, or as dose corrected for body weight (which is greatest between *B. terrestris* and *O. bicornis* than for any other species pair) this scale of interspecies variation in sensitivity found for dose is not repeated and species sensitivity for cadmium then becomes bound within an order of magnitude variation.

The only other case where toxicity showed large potential inter-species variation was for tau-fluvalinate; there was no toxicity of tau-fluvalinate even at the highest tested concentrations for *A. mellifera*, which was related to the maximum water solubility of this chemical, while for both *B. terrestris* and *O. bicornis* effects on survival were seen that allowed LC_{50} values to be estimated. These LC_{50} values for these two species were generally in the range from 30–150 µg/L depending on species and exposure time. The NEC that can be estimated for tau-fluvalinate of 37.8 µg/L and 12.5 µg/L for *B. terrestris* and *O. bicornis* respectively compared with maximum concentration used of 900 µg/L in the *A. mellifera* tests that did not result in an effect on survival in the experiment. Hence, for tau-fluvalinate the ratio of species sensitivity between *A. mellifera* and the other two tested species

will be in the range of 30 for *B. terrestris* and 75 for *O. bicornis*. Between *B. terrestris* and *O. bicornis* comparisons of probit calculated LC_{50} values indicate initially similar sensitivity, but suggest that *O. bicornis* may be up to 50 fold more sensitive at 240 h exposure. Probit model fit for the tau-fluvalinate study are particularly weak as a result of the relatively high control mortality in this test. Hence a better comparison of sensitivity of these species is made by the DEBtox NEC. This suggests that *O. bicornis* may be three fold more sensitive to tau-fluvalinate than *B. terrestris*. The low sensitivity of *A. mellifera* to tau-fluvalinate has been previously linked to the high metabolic potential for this insecticide in this species (Johnson et al., 2013). Results in this current study for the other two tested species suggest that there may be a lower metabolic potential in the other two species with the result that sensitivity for this pyrethroid is greatly increased.

Table 8: Comparative sensitivity of the three bee species expressed as the ratio of LC_{50} values for each species calculated by either probit analysis for effects on survival at 96 h or using DEBtox parameters from the best fitting model for three time points

A) 48 h exposure time

	Probit analysis			**DEBtox parameters**		
	Apis:Bombus	*Apis:Osmia*	*Bombus:Osmia*	*Apis:Bombus*	*Apis:Osmia*	*Bombus:Osmia*
LC_{50} (mg/L)						
Dimethoate	1.11	0.31	0.28	0.94	0.14	0.15
Clothianidin	2.74	2.48	0.90	1.57	0.80	0.51
Tau-fluvalinate	NC	NC	1.24	1.93	0.22	0.11
Propiconazle	NC	NC	NC	NC	NC	NC
2,4-D	NC	NC	NC	NC	NC	NC
Cadmium	0.82	0.67	0.82	1.70	0.67	0.39
Arsenic	1.21	0.51	0.42	1.14	0.38	0.33
LD_{50} (mg/bee)						
Dimethoate	0.22	0.22	0.99	0.19	0.10	0.52
Clothianidin	0.55	1.73	3.17	0.31	0.56	1.77
Tau-fluvalinate	NC	NC	NC	0.39	0.15	0.39
Propiconazle	NC	NC	NC	NC	NC	NC
2,4-D	NC	NC	NC	NC	NC	NC
Cadmium	0.16	0.47	2.87	0.34	0.47	1.38
Arsenic	0.24	0.35	1.47	0.23	0.27	1.16
LD_{50} by body weight (mg / mg bee)						
Dimethoate	0.44	0.16	0.37	0.38	0.07	0.19
Clothianidin	1.09	1.30	1.19	0.63	0.42	0.66
Tau-fluvalinate	NC	NC	NC	0.77	0.11	0.15
Propiconazle	NC	NC	NC	NC	NC	NC
2,4-D	NC	NC	NC	NC	NC	NC
Cadmium	0.33	0.35	1.08	0.68	0.35	0.52
Arsenic	0.48	0.27	0.55	0.46	0.20	0.44

B) 96 h exposure time

	Probit analysis			DEBtox parameters		
	Apis:Bombus	*Apis:Osmia*	*Bombus:Osmia*	*Apis:Bombus*	*Apis:Osmia*	*Bombus:Osmia*
LC_{50} (mg/L)						
Dimethoate	1.13	0.44	0.39	1.31	0.17	0.13
Clothianidin	2.20	1.77	0.81	1.80	0.69	0.38
Tau-fluvalinate	NC	NC	55.34	3.41	0.43	0.13
Propiconazle	NC	NC	NC	NC	NC	NC
2,4-D	NC	NC	NC	NC	NC	NC
Cadmium	0.38	1.68	4.41	1.75	1.01	0.58
Arsenic	1.56	4.39	2.81	0.91	0.45	0.49
LD_{50} (mg/bee)						
Dimethoate	0.23	0.31	1.36	0.26	0.12	0.44
Clothianidin	0.44	1.24	2.82	0.36	0.48	1.34
Tau-fluvalinate	NC	NC	NC	0.68	0.30	0.44
Propiconazle	NC	NC	NC	NC	NC	NC
2,4-D	NC	NC	NC	NC	NC	NC
Cadmium	0.08	1.17	15.43	0.35	0.71	2.03
Arsenic	0.31	3.07	9.82	0.18	0.31	1.72
LD_{50} by body weight (mg / mg bee)						
Dimethoate	0.45	0.23	0.51	0.52	0.09	0.17
Clothianidin	0.88	0.93	1.06	0.72	0.36	0.50
Tau-fluvalinate	NC	NC	NC	1.36	0.23	0.17
Propiconazle	NC	NC	NC	NC	NC	NC
2,4-D	NC	NC	NC	NC	NC	NC
Cadmium	0.15	0.88	5.79	0.70	0.53	0.76
Arsenic	0.63	2.30	3.68	0.36	0.23	0.64

C) 240 h exposure time

	Probit analysis			DEBtox parameters		
	Apis:Bombus	*Apis:Osmia*	*Bombus:Osmia*	*Apis:Bombus*	*Apis:Osmia*	*Bombus:Osmia*
LC_{50} (mg/L)						
Dimethoate	1.71	NC	NC	2.57	0.27	0.11
Clothianidin	1.02	0.60	0.59	2.17	0.54	0.25
Tau-fluvalinate	NC	NC	NC	5.74	1.06	0.19
Propiconazle	NC	NC	NC	NC	NC	NC
2,4-D	NC	NC	NC	NC	NC	NC
Cadmium	0.15	0.83	5.50	1.45	2.05	1.41
Arsenic	0.92	NC	NC	0.74	0.67	0.92
LD_{50} (mg/bee)						
Dimethoate	0.34	NC	NC	0.51	0.19	0.37
Clothianidin	0.20	0.42	2.05	0.43	0.38	0.88
Tau-fluvalinate	NC	NC	NC	1.15	0.74	0.65
Propiconazle	NC	NC	NC	NC	NC	NC
2,4-D	NC	NC	NC	NC	NC	NC
Cadmium	0.03	0.58	19.25	0.29	1.43	4.93
Arsenic	0.18	NC	NC	0.15	0.47	3.20
LD_{50} by body weight (mg / mg bee)						
Dimethoate	0.68	NC	NC	1.03	0.14	0.14
Clothianidin	0.41	0.31	0.77	0.87	0.28	0.33
Tau-fluvalinate	NC	NC	NC	2.30	0.56	0.24
Propiconazle	NC	NC	NC	NC	NC	NC
2,4-D	NC	NC	NC	NC	NC	NC
Cadmium	0.06	0.44	7.22	0.58	1.07	1.85
Arsenic	0.37	NC	NC	0.29	0.35	1.20

Species sensitivity can also be compared by comparison of the NEC derived from the DEBtox fits (Table 9). Unlike LC_{50} estimates, the NEC provide a time independent comparison of sensitivity that is not also affected by toxicokinetic and toxicodynamic parameters. Estimates of the NEC values indicate that there is a larger range of interspecies variation than found by probit analysis. Only three of the interspecies comparisons indicate that comparative sensitivity is within a factor of two. For the remainder of comparisons, NEC values are within a factor of 10 between species for 11 of 15 inter-species comparisons. The exceptions to this, are comparisons for tau-fluvalinate between *A. mellifera* and the other two tested species and for cadmium. For tau-fluvalinate, differences in sensitivity between species were also found when values were estimated by probit fits. Species differences here may be related to variations in the metabolic capacity of the tested species for the pyrethroid and at this magnitude, may be an important additional consideration for the compound in cases where there may be exposure of wild bee species. The variation in the NEC calculated for cadmium arose in part because of the very low estimated parameter values for this metal. Because the NEC is a value estimate independent of exposure time, it is possible that when toxicokinetics are slow the NEC may not be reached in a biologically relevant timeframe. For cadmium, the comparison of NEC, while theoretically valid may not relate to actual sensitivity for realistic relevant exposure time, as these will be limited by life-span. Analysis of effects over the duration of the study and for time beyond (e.g. average worker summer life-time of 720 h) may in this specific case be a better comparison. The ratios of species sensitivity for LC_{50} and LC_5 value are: 0.18 and 0.85 for *A. mellifera : B. terrestris*; 6.01 and 6.04 for *A. mellifera : Osmia* and; 31.7 and 7.1 for *B. terrestris* and *O. bicornis* respectively. Hence, realistic estimates of sensitivity indicate variation within a factor of 10 except for LC_5 effects between *B. terrestris* and *O. bicornis* species.

3.3.4. Comparison of lethal and sub-lethal effects

In addition to monitoring effects on survival, the effects of exposure on bee behaviour were recorded. Behaviours were scored to identify changes such as erratic movement patterns, shaking, lethargy and aberrant response to stimulus. Individuals showing such behaviour were scored as affected (binary; 1

for affected, 0 for not affected), to establish the total number of impaired bees in each replicate container as a proportion of those entering the test. This was not done for all tests in the current study so exemplar data are presented from responses of species to clothianidin exposure alone, in mixture tests using this chemical. Recording of impairment identified a clear pattern of response; behaviour effects are often detected at concentrations earlier during the exposure compared to mortality i.e. bees may be impaired and unable to function at time points significantly before mortality is recorded (Figure 8). This lag in terms of inability to function before mortality could be 24 h–48 h for *A. mellifera* and *O. bicornis*, but could be longer in *B. terrestris*. As a result the proportion of test individuals showing behavioural effects (total impaired compared to dead) was often higher in *B. terrestris* than the other two species. In bees with altered behaviour, the delay frequently observed between the onset of behavioural response and lethality may be a consequence affected bees being unable to access food and therefore an additional, ultimately lethal, exposure. This effect may be greater for *B. terrestris* and *O. bicornis* which do not show trophallaxis. During this time when feeding is not possible in non-*Apis* species, it is feasible that there may even be recovery in condition as a result of endogenous metabolism. At the point where bees show sufficient recovery, they may then be able to reach the feeder to allow further sucrose ingestion that supplies a further, potentially lethal, dose.

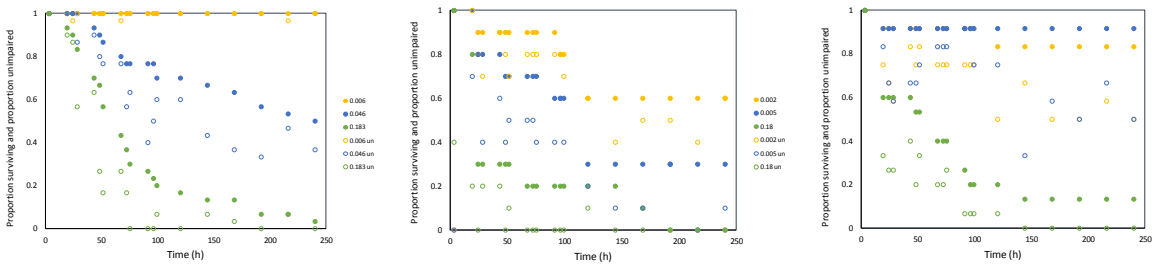


Figure 8: Proportion survival (filled circles) and sub-lethal unimpaired (open circles) patterns in time for populations of *A. mellifera* (right), *B. terrestris* (middle) and *O. bicornis* (right) exposed to a low, medium and high toxicity concentrations clothianidin

To compare the sensitivity of the behavioural endpoint, effect concentrations (EC_{50}) for changes in behaviour were calculated for the 48 h, 96 h and 240 h time point of the experiment. The values were compared to the LC_{50} values for the sample time points to provide an indication of the comparative sub-lethal sensitivity. This was expressed either as a ratio of the LC_{50} and EC_{50} values for behavioural effects. Sub-lethal effects were between 1.5 and 2.5 fold more sensitive than measurement of effects on survival alone (Table 9). The increased sensitivity of sub-lethal measurement was greatest for *B. terrestris* than the other two species. This may be related to a number of traits of this species including more intermittent feeding and also larger body size which may allow the larger bumblebee to survive for longer without feeding survival than in honeybee or solitary bees. Additionally for *A. mellifera*, there is the possibility of continued exposure of impaired bees through tropholaxis.

The obvious effects on behaviour that we measured represent a range that is characteristic of severe intoxication. There is a growing body of information available on methods for determining more subtle behavioural traits linked to foraging behaviour, hive location and social interactions. For chemical eliciting such effects, it may be expected that this would occur at concentrations below those causing the gross behavioural effects observed in this study (Godfray et al., 2014; Lundin et al., 2015; Sol Balbuena et al., 2015; Stanley et al., 2015; Zhang and Nieh, 2015).

Table 9: LC_{50} and EC_{50} values for effects of clothianidin only exposure on behaviour and the ratio between them as an indication of the comparative sensitivity of sub-lethal effect assessment using behaviour for the three bee species.

	LC_{50} µg/L	EC_{50} µg/L	LC_{50} : EC_{50}
48 h			
Apis mellifera	0.219	0.148	1.48
Bombus terrestris	0.0244	0.0125	1.95
Osmia bicornis	0.112	0.069	1.62
96 h			
Apis mellifera	0.128	0.071	1.80
Bombus terrestris	0.0174	0.007	2.49
Osmia bicornis	0.106	0.0549	1.93
240 h			
Apis mellifera	0.069	0.0511	1.35
Bombus terrestris	0.0156	0.0069	2.26
Osmia bicornis	0.0555	0.036	1.54

3.4. Phase 4 – Mixture toxicity experiments

3.4.1. Overall design and interpretation of mixture studies

The two experimental design used allowed assessment of chemical interactions affecting both of single chemical toxicity in the presence of an anticipated non-toxicity concentration of a second chemical (Potentiation tests) or the joint effects of two toxic chemicals (Mixture toxicity test). The different designs demanded the use of different data analysis approaches. Potentiation studies are amenable to classic analysis using single chemical concentration response curves. The mixture toxicity tests were analysed by effect estimation according to CA and independent action using the MIXTOX model. Both designs are amenable to analysis using appropriate DEBtox tools which can be fitted initial as models that describe the effects of the two single chemicals and, subsequently by extension the mixture. The designs used, thus, allow the comprehensive analysis of joint effect measured through classic statistical approach supported by the additional mechanistic assessment integrated through fitted DEBtox models. The patterns for mixture effects for each species and chemical combination tested are set-out below.

3.4.2. *Apis mellifera* adults

Potentiation experiments

Dimethoate potentiated by propiconazole:the 48 h, 96 h and 240 h LC_{50} values for dimethoate were consistent with the previous single chemical study. No effect of propiconazole alone was found for any timepoint as was expected. Calculated LC_{50}s for dimethoate were similar across all time points independent of propiconazole level. DEBtox model NECs for dimethoate in absence and presence of were almost identical and the toxicokinetic and toxicodynamic parameter values closely related in single and mixture studies. This suggests independence of dimethoate toxicity from fungicide addition with no evidence of potentiation. This was further supported as the DEBtox fit for mixture was not improved by inclusion of an additional parameter allowing for between chemical interactions.

Clothianidin potentiated by propiconazole: time dependent trends of clothianidin toxicity were found that were consistent with those found in the single chemical studies. No direct effect of propiconazole was found. Clothianidin LC_{50}s in the absence and presence of propiconazole were always within a factor of 2, although lowest values were always found in the high fungicide treatment for each timepoint. The DEBtox model fits for clothianidin alone gave similar parameter values to those found for the initial single chemical studies. Addition of propiconazole had only small effects on DEBtox parameter values. Values for the NEC were almost identical and the killing and elimination rates show very close alignment across the three treatment series. This indicates at most only a small influcence of propiconazole on clothianidin toxicity.

Clothianidin potentiated by tau-fluvalinate: time dependent trends for clothianidin toxicity were consistent with those found in both the propiconazole and single chemical studies. No direct effect of tau-fluvalinate was found as was anticipated. Calculated clothianidin LC_{50} values indicated a higher sensitivity in the presence of high concentration of tau-fluvalinante at 48 h, but values were similar in all treatments at all later timepoints. The DEBtox model NEC differed by a factor of 2 in the test conducted in the presence of high tau-fluvalinate concentrations compared to the exposure without the pyrethroid. The DEBtox elimination rate was reduced approximately 4 fold from the study without tau-fluvalinate compared to the high tau-fluvalinate concentration test (the low tau-fluvalinate concentration was intermediate). This suggests a possible effect of tau-fluvalinate on clothianidin toxicokinetics that may slightly alter the progression of toxicity in time.

Mixture Toxicity experiments

Dimethoate and clothianidin mixture: analysis of the joint effect of clothianidin and dimethoate was conducted using the MIXTOX joint effect modelling framework for the 48 h, 96 h and 240 h survival data (Jonker et al., 2005). Both CA and IA provided a very significantly enhanced fit to the data for all timepoints compared to null model of no joint effects. Addition of the S/A parameter significant improved both CA and IA based model fit for the mixture. The value of *a* in the extended model was greater than 1 indicated that the interaction was predominantly antagonistic. DEBtox model of the effects data identified slight over-prediction of toxicity for the model that does not allow for an interaction component. Including a parameter that allowed for an antagonistic interaction improved the revised model fit compared to the un-amended model.

Clothianidin and Cd mixture: CA and IA model fits for time-points at 96 h and 240 h were highly significant against a model of no joint effect. Addition of a further model parameter through the CA Model identified a significant antagonistic interaction at 240 h, but not 96 h. The value for *a* was > 1 suggesting antagonism. Inclusion of the b_{DL} parameter significantly improved the model fit for the 240 h data, with the parameter value indicating that the interaction was greater at high Cd concentrations. Addition of parameters to the basic IA models failed to significantly improve the model fits for any time-points. This suggests that the joint effects of the two chemical may be jointly additive according to the independence assumptions of IA. DEBtox fits resulted in model parameters in close agreement with single chemical test results. The model was not improved by inclusion of an interaction parameter supporting the absence of any interaction in this mixture.

As and Cd mixture: CA and IA fits were highly significant against a model of no joint effect. IA provided a slightly better fit to the data that the CA model, although this difference was only small Inclusion of interaction parameters failed to improve the fit of the model to the observed data for both CA and IA for all time-points. This suggests an additive joint effect for the two trace elements across the different time points. Values for the DEBtox fits were consistent with those from the earlier single chemical study. Fits for each metal were not affected by co-exposure. Inclusion of an interaction parameter did not enhance the model fits. This suggests an independent effect of the two elements across the full exposure.

3.4.3. *Apis mellifera* larvae

Potentiation experiments

Dimethoate potentiated by propiconazole: exposure of *A. mellifera* larvae to dimethoate alone resulted in a significant effect on mortality only at the highest tested concentration and dose of 23.8 µg/bee. Co-exposure with propiconazole resulted in a greatly elevated mortality in this top treatment which may suggest a slight synergising of the organophosphate by the fungicide. In the presence of propiconazole, an EC_{50} for dimethoate for effects on larval weight of 1.2 µg/bee was three fold lower than that for dimethoate alone. Variation in the larval weight data means that the uncertainty associated with these point estimates is high. Hence, operationally the response curves for the two exposure series cannot be separated and without further evidence, the joint effects of the two chemicals should be viewed as nominally independent.

Clothianidin potentiated by propiconazole: exposure of larvae to clothianidin alone did not result in any significant dose related effect on larval survival over the 72 h post exposure. The presence of propiconazole did not elicit an effect on survival due to clothianidin at any time-point. Hence, it appears that not only is clothianidin alone unable to elicit an effect on survival at the tested concentration, but that propiconazole does not potentiate any such effect. Clothianidin significantly reduced larval weight at doses of 0.32 µg/bee and higher. This sub-lethal effect of clothianidin was not affected by propiconazole co-exposure indicating no potentiation or inhibition.

Clothianidin and tau-fluvalinate: no effect of clothianidin on larval survival was found for any treatment, as was also found in the study conducted with propiconazole. Further, the absence of an effect on mortality was also seen for exposures conducted in the presence of tau-fluvalinate. This suggests that the two chemicals show independent effects that are not characterised by the potentiation of toxicity. In the presence of tau-fluvalinate, larval weight was reduced at the two top clothianidin concentrations of 0.84 and 2.19 µg/bee. No effect of clothianidin alone on larval weight was found. When exposed with tau-fluvalinate, larval weights were significantly reduced at the top two concentrations. This toxicity at these higher treatments may point to a slight potentiation of clothianidin effect by tau-fluvalinate.

3.4.4. *Bombus terrestris* adults

Potentiation experiments

Clothianidin potentiated by propiconazole: exposure of clothianidin alone for *B. terrestris* identifies a progressive decrease in the LC_{50} over time that is consistent with the result of the initial single chemical exposures. Robust model fits for *B. terrestris*, identified a small potentiating effect of propiconazole on clothianidin toxicity. This resulted in a reduction in the LC_{50} value for clothianidin of 1.5 to 2 fold in the exposure in the presence of low and high propiconazole concentrations. DEBtox fits indicated that addition of propiconazole to the mixture results in a small change in the NEC and elimination rate. The change in the NEC that is predicted, suggests a higher sensitivity in the presence of the fungicide in a manner that is consistent with the changes seen in LC_{50} values. Hence the DEBtox analysis points to a similar slight potentiation of clothianidin toxicity by propiconazole.

Clothianidin potentiated by tau-fluvalinate: patterns of the effects of clothianidin alone were consistent with those from both the single chemical tests and the study with propiconazole. When exposure was with tau-fluvalinate, a small potentiating effect on clothianidin toxicity was indicated. At 48 h this small (< 1.5 fold) effect was seen in both the low and high concentration tau-fluvalinate treatment, although at 96 h and 240 h it remained only for the high tau-fluvalinate exposure. The tau-fluvalinate exposure alone was, however, found to cause a direct toxic effect at the higher test concentration. Hence the increase effect observed in the mixture treatments appears to be driven by a direct tau-fluvalinate effects rather than a potentiation of clothianidin toxicity by the pyrethroid. A single reliable DEBtox model could not be derived for clothianidin effects on survival. Fits instead gave a range of plausible parameter values in respect of the NEC, toxicokinetics and toxicodynamics. This range of model fits generally gave parameter values in the better fitting models that were in the range of the values for the single chemical data for 2015 resulted in parameter values that were in close agreement with the single chemical and propiconazole model fits. Model fits were not enhanced by inclusion of an interaction parameter supporting our conclusions that clothianidin and tau-fluvalinate the two chemical both contribute to observed toxicity and that effects are independent and additive rather than interactive through potentiation.

Mixture Toxicity experiments

Dimethoate and clothianidin mixture: MIXTOX models for all timepoints indicated a high significance of both the CA and IA model against the null hypothesis of no joint effects. CA provided a better description of the data than IA (r^2 0.71 CA, compared to r^2 0.53 IA). Addition of the S/A parameter to the baseline models significantly improved model fit for both CA and IA. The parameter value for *a* was greater than one, indicating antagonism. Addition of the DL and D parameters to both models

further improved model fits. Parameter values indicated greater antagonism in mixtures where dimethoate is dominant in the mixture. Within DEBtox models, the NEC values derived for model fits were within a factor of 2 for both chemicals indicating no systematic sensitivity shift in the mixture exposures. The toxicodynamic parameter as the killing rate were also closely aligned. There was however a difference in elimination rates between tests that describe a different time course for the observed toxicity, but no change in the absolute magnitude of the effect. We conclude that the joint effect of dimethoate and clothianidin in *B. terrestris* is dominantly antagonistic with subtle dose level and ratio effects and that these interactions are determined by changes in toxicokinetic traits.

3.4.5. *Osmia bicornis* adults

Potentiation experiments

Dimethoate potentiated by propiconazole: probit model fits for *O. bicornis* were weaker than those for *A. mellifera* as indicated by the larger 95% confidence intervals for calculated effect concentrations. Dimethoate exposure alone and in the presence of low and high propiconazole concentrations gave similar LC_{50} values. This suggests no potentiation of dimethoate toxicity by the fungicide. Robust DEBtox fits for male *O. bicornis* gave lowest NECs in the exposure conducted in the presence of low and high propiconazole. The small, but possibly important variations in elimination rate was found, with values decrease by 2 and 3 fold in the presence of low and high propiconazole concentration. The killing rate is also changed approximately 10 fold between exposures, although in this case, the values are higher in the exposure with propiconazole. The overall model fits for *O. bicornis* suggest that there is no systematic effect on sensitivity to propiconazole, but that there may be some difference in toxicokinetics and toxicodynamics that may affect the overall time course of effect in co-exposure. Robust DEBtox model for female bees could not be derived, with numerous parameter combinations giving almost equally good fits. The majority of combinations suggest a NEC of 0.5 to 1.1 mg/L for the experiment in the absence and presence of propiconazole. This suggests that there is no effect of propiconazole sensitivity to dimethoate in female *O. bicornis*. Hence, the DEBtox analysis suggests possible sex specific differences in the interaction between the two chemicals.

Clothianidin potentiated by propiconazole: clothianidin exposure alone and in the presence of low and high propiconazole concentrations identified a trend for reduction in the LC_{50} values with increasing concentrations of the fungicide. This difference being up to 2 fold in the high propiconazole treatment. For males, it was not possible to derive reliable DEBtox models for the exposure in the absence or presence of propiconazole. A range of similar model fits gave almost equally good descriptions of the data. The majority of these different plausible model fits place the NEC between 0.02 and 0.05 µg/ml and do not identify an effect of propiconazole on sensitivity. For females, models also provide a range of plausible fits. Possible NECs show no consistent variations related to the presence of propiconazole, although the large variation in possible parameter values may mask identification of potentiation of LC_{50} values observed from single time point probit analysis.

Mixture Toxicity experiments

Dimethoate and clothianidin mixture: mixture model fits for males for both CA and IA gave a significant improved fit compared to the alternative hypothesis of no joint effect for male but not female bees nor for the combined data-set. The improved model fit for males for the mixture following addition of the S/A parameters was positive for both models, although the fits of the overall models was relatively week. This was due both to the limited number of individuals that could be tested peer treatment and also the absence of a high dose effect on males from clothianidin exposure. The positive value of *a* in the model that could be fitted for males, indicates primarily antagonism in relation to both CA and IA predictions. Further extension of the model by inclusion of the DL and DR parameters indicated that the antagonism indicated for male *O. bicornis* was greatest at high effects levels and in mixture treatments where the toxicity of clothianidin was dominant. For females, amendment of models with the parameter to describe S/A or DL or DR parameters did not significant improve the fit of the data to either the CA or IA model. This was also the case when the male and female data-sets were combined and analysed. Thus, in contrast to males the effects of the two

chemicals are additive for females. Reliable DEBtox models for male *O. bicornis* could not be fitted as a number of plausible parameter sets could equally well describe the time-series effects. Models suggested a likely dimethoate NEC of around 0.2 µg/ml with no effect of the presence of clothianidin. For clothianidin, a reliable NEC could not be derived. There was an indication of an effect on the toxicokinetics of dimethoate, with effects reduced as the toxicokinetic and toxicokinetic and toxicodynamic parameters in the model. However, this effect is based only on weakly fitting models and so requires further validation. For the female *O. bicornis*, a good model fit could be generated. The NEC for the effects of dimethoate was not affected by clothianidin exposure. A model for the effects of clothianidin could not be fitted due to the absence of an effect of the neonicotinoid at the highest tested concentration. No effect of clothianidin on dimethoate toxicity was evident. Hence, DEBtox alone does not point to a strong interaction in the mixtures.

3.4.6. Overall patterns of joint effect

In the potentiating studies, the concentration series used showed concentration dependent effects on survival with the further benefit that they largely showed progression over time. Within these tests, a modulating effect of the potentiating chemical on the toxic chemical would be seen by a shift of the concentration response curve to the left (potentiation) or right (alleviation) along the concentration axis. Results pointed to only a limited degree of interaction relating to a synergistic or antagonistic experiment across the range of combinations and species tested as summarised in Table 10.

For two potentiating mixtures, namely dimethoate and propiconazole studied in *A. mellifera* and *O. bicornis* and clothianidin and tau-fluvalinate in *A. mellifera* and *B. terrestris* did not identify any effect of the potentiating chemical on the primary toxicant in any case. For these two mixtures, this suggests that the joint effects of the two chemicals tested in combination are independent at the concentrations jointly studied.

For the studies that assessed the toxicity of clothianidin in the absence and presence of propiconazole some evidence suggesting potentiation was found. For *A. mellifera* any effect was relatively weak being associated with a maximum 1.5 fold change in the LC_{50} values derived from studies with and without the fungicide present (Figure 9 a–c). This was also the case for the *A. mellifera* larval test for clothianidin in the presence of propiconazole for which a strongly potentiating effect was not evident. For *O. bicornis* and *B. terrestris*, the potentiation seen was somewhat more pronounced, being up to 2 fold, and was also stronger in the treatments with a higher propiconazole present.

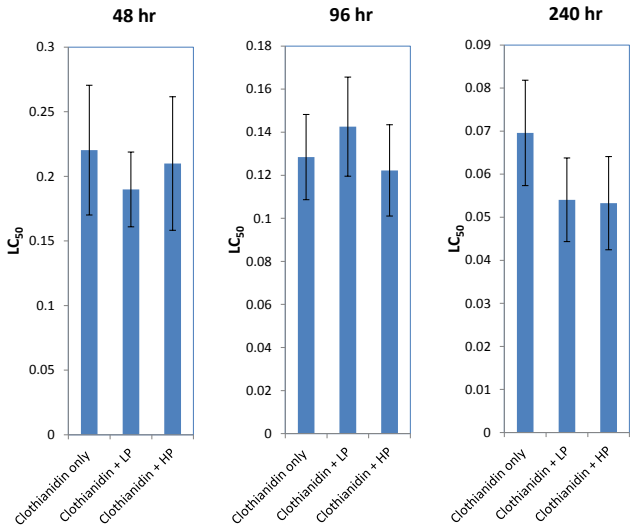
Table 10: Summary of the nature of interactions identified in the joint effects of binary combinations of chemicals in Potentiation and Mixture Toxicity experiment conducted with three bee species.

	96 h CA	96 h IA	DEBtox
Apis mellifera			
Dimethoate + propiconazole	No potentiation	No potentiation	No potentiation
Clothianidin + propiconazole	Slight potentiation	Slight potentiation	No potentiation
Clothianidin + tau-fluvalinate	No potentiation	No potentiation	No potentiation
Dimethoate + clothanidin	Antagonism	Antagonism	Partial antagonism
Clothianidin + Cd	Slight antagonism	Additive	No interaction
Cd + As	Additive	Additive	No interaction
Osmia bicornis			
Clothianidin + propiconazole	Moderate potentiation	Moderate potentiation	Slight potentiation
Clothianidin + tau-fluvalinate	No potentiation	No potentiation	No potentiation
Dimethoate + clothanidin	Antagonism ♂ only	Antagonism ♂ only	Partial antagonism
Bombus terrestris			
Dimethoate + propiconazole	No potentiation	No potentiation	No potentiation
Clothianidin + propiconazole	Moderate potentiation	Moderate potentiation	Slight potentiation
Dimethoate + clothanidin	Antagonism	Antagonism	Partial antagonism

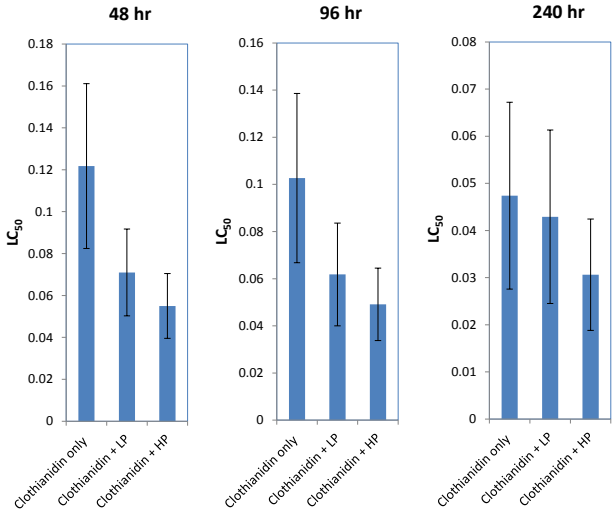
Although the comparison of the response curves for the clothianidin mixture suggests some potentiation, this is only partially supported by the DEBtox analysis. DEBtox models for *A. mellifera* do not suggest any clear potentiation across the full experiment. An interaction between the two chemicals is indicated for the *B. terrestris* and *O. bicornis* models, including through changes in the NEC, as well as the toxicokinetic and toxicodynamic parameters. If any one of the species studies had shown a potentiation effect of the scale and certainty indicated by the response curve and DEBtox analysis, then these may be seen as uncertain in relation to their biological meaning. Because, however, the same indication of potentiation in seen across species and with different analysis tools, the potential for these interaction to have a mechanistic basis is better supported.

The magnitude of the potentiation effects of propiconazole on clothianidin that was seen was in the range of a maximum of approximately two fold in *O. bicornis* down to only 1.5 fold in *A. mellifera* for early time points (and absent at later times). The magnitude of such a change do not mirror the orders of magnitude changes that have been previously reported for studies between tau-fluvalinate and sterol biosynthesis inhibiting fungicides (Johnson et al., 2013). These large magnitude interactions with the pyrethroid and fungicide were attributed to inhibition of the cytochrome P450 system by the different fungicides tested which prevented the rapid detoxification of the tau-fluvalinate by honeybees. The absence of such a strong potentiation effects of a representative sterol inhibiting fungicide on clothianidin toxicity may reflect differences in the nature of metabolism of different insecticide groups between bee species. However, given that the effects, although small in magnitude is common between species, the potential for further potentiation assessments of insecticide toxicity assessment with sterol inhibiting fungicide may be important to consider in further work.

A) *Apis mellifera*



B) *Osmia bicornis*



C) *Bombus terrestris*

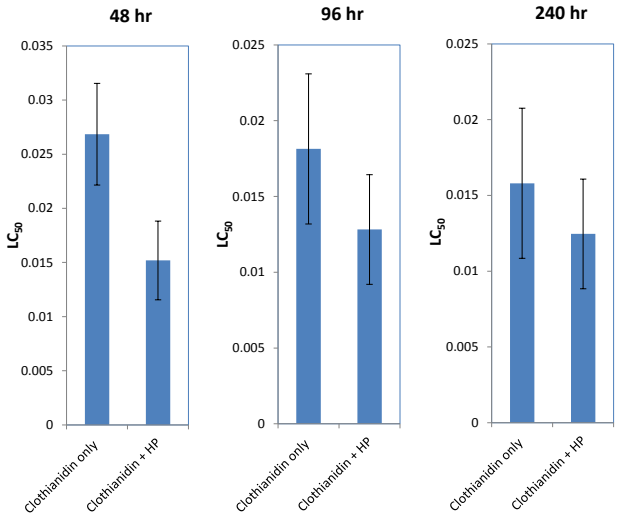


Figure 9: LC_{50} for *Apis mellifera* (Top 3 panels), *Bombus terrestris* (middle 3 panels), and *Osmia bicornis* (Bottom 3 panels) exposed to clothianidin in the presence of no, low or high concentrations of propiconazole at exposure times of 48 h (Panel 1 of 3), 96 h (Panel 2 of 3) and 240 h (Panel 3 of 3), Values are $LC_{50} \pm 95\%$ confidence interval

The mixture toxicity experimental design was used for those binary mixtures where both chemicals showed a clear concentration response for effects on mortality in previous single species tests. The experimental set-up was based on the CA concept and was specifically tailored to include equitoxic mixtures at different concentrations as well as different mixture ratios, although inclusion of single chemical treatments and at the same levels as used in the mixture treatment also allows data analysed IA (see Figure 1). This overall design allow application of the MIXTOX model framework to identify statistically significant interactions in mixture tests and to establish the maximum magnitude of such effects both from an effect level change and concentration fold change perspective (Martin et al., 2009; Svendsen et al., 2010). In all cases, the fits of the CA and IA model provided a better description of the data that the null model of no joint effect. This finding is entirely in accordance with expectations for models that have a long history of use across a range of joint chemical stressor exposure cases. In almost all cases, the CA and IA model fits gave very similar fits. It has been stated previously that at least for binary mixtures, it is difficult to operational assess the validity of the two models from a mechanistic context based on goodness of fits parameters only. In effect, it is absence of specific information, each can be considered as equally valid. Further work to specifically analyses the AOPs related to the toxic effects of each chemical in the tested species would be needed to understand their mechanistic effects and thereby the most biologically valid mixture model.

The model fits for the three mixture combination in *A. mellifera* identified different patterns of response. For the cadmium and arsenic mixture, no interaction was indicated for either model at all time-points. This suggests that the joint effects of these two chemicals are additive without interaction. For the clothianidin and cadmium mixture, fits of models based on CA identified possible antagonistic interactions. However, the IA fits indicated additivity. Given the different modes of action of the pesticide and metals, it is likely that this mixture is additive based on the IA principal of independent modes of action. The final mixture toxicity combination tested dimethoate and clothianidin, was found to show an interaction characterised by antagonism particularly at high exposure concentration in cases where dimethoate in dominant in the mixtures. This antagonism was observed for *A. mellifera* and *B. terrestris* (Figure 10). A significant antagonistic interaction was also found for male *O. bicornis*, although from a weakly fitting overall model (Figure 10). Such an interaction was not, however, found for female or the combined male female data-set indicating possible sex specific difference in the responses of this species to joint exposure to these two insecticides. The overall magnitude of the antagonistic interactions observed in the experiments with

dimethoate and clothianidin were not large in magnitude of effect (see Figure 10). However, importantly the same response pattern was also seen for the mixture toxicity studies with male *O. bicornis*, *A. mellifera* and *B. terrestris*. This was characterised dominantly by antagonism which means that when plots of observed versus predicted effects are drawn, the observed data for mixture is usually below the effect predicted by the CA model (Figure 10). These deviations tend to be slightly greater in mixtures where clothianidin is dominant in the mixture. Given this similarity of response pattern, it is likely that the indicated antagonism in each species may be biologically meaningful.

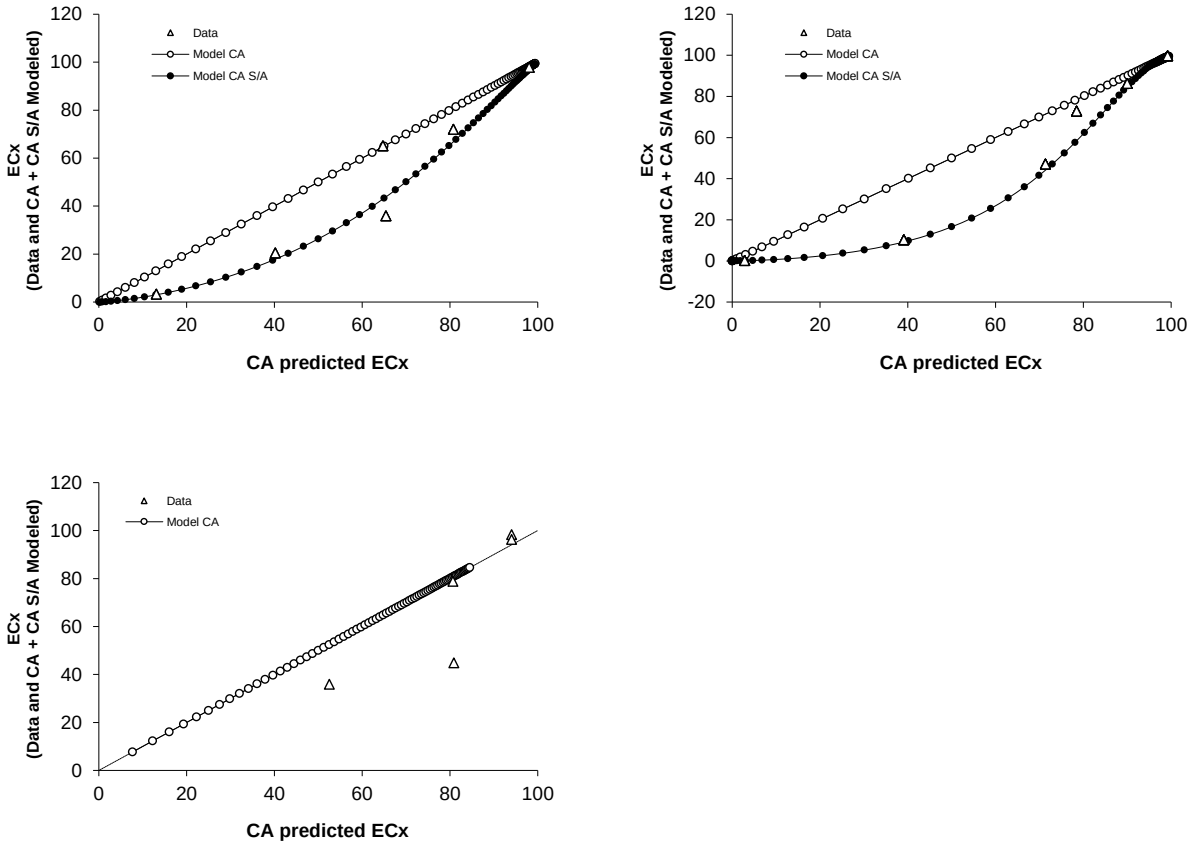


Figure 10: Fitted concentration addition models and observed data for the effects of single treatment of dimethoate and clothianidin and their mixtures on survival rate of adult *A. mellifera* (top), *B. terrestris* (middle) and *O. bicornis* male bees (bottom) exposed for 96 h by continuous feeding with fitted line corresponding to CA model predictions and point corresponding to observed mixture effect data

Clothianidin and dimethoate in the antagonistic mixture are both known to be metabolised by the insect cytochrome P450 systems. However, the nature of this metabolism and its consequence for toxicity differs between the compounds. Dimethoate is metabolically activated, while clothianidin in metabolically detoxified. A further consideration is that although supplied at similar effect levels (0.25 TU, 0.5 TU etc.) in the mixture exposure, the actual concentration of dimethoate will exceed those of clothianidin by at least an order of magnitude due to the lower potency of the organophosphate. If the amount of active cytochrome P450 in the mixture exposed bee remains the same and the two chemical have equal affinity, then we may expect dimethoate to preferentially be metabolised. This would mean that the more potent neonicotinoid may not be metabolised, which we would expect to result in synergism compared to the non-mixture case where access to cytochrome P450 is not inhibited. If, however, the higher concentrations of dimethoate induce greater cytochrome P450 isozyme expression and clothianidin is a favoured substrate, then this may result in higher

neonicotinoid metabolism leading to a reduced toxicity for the mixture. Such subtle mechanisms may be at play in determining the mixture interaction commonly observed between the tested species.

4. Conclusions

4.1. Phase 1 – Test design and optimisation

- Three bee species were selected for study. These were the euscocial domesticated honeybee *A. mellifera*, the colonial bumblebee *B. terrestris* and the solitary bee *O. bicornis*. The three species cover a range of possible taxa, habits, social traits, body sizes and physiologies providing a test case to assess the range of variation in bee sensitivity to the different chemicals selected for study.
- The experimental design developed and used for both *A. mellifera* and *B. terrestris* adult tests provided an excellent platform within which to assess single chemical mixture acute toxicity over exposure durations currently recommended in the existing OECD toxicity tests guideline for oral exposure in honeybees (OECD, 1998a). In all tests conducted with these two species, control mortality was < 80% (the desired value for test validity within relevant OECD test guidelines) for the single chemical and mixture tests after 96h. Extended exposure to 240 h resulted in control mortality also < 80% in all tests conducted except one.
- *O. bicornis* were obtained directly from supplies collected from field populations as pupae. On receipt, the pupae were refrigerated for at least four months allowing the extension of the testing period for this species from March until July 2015. Both male and female *O. bicornis* responded well to feeding of un-dosed and dosed sucrose solutions but despite this, in the first year of study for the single chemical tests, control mortality was often high for this species (up to 50% in some tests). Further trials using different feeding regimes failed to alleviate this effect. Instead a cohort effect was consistently noted whereby one portion of the population was lost early during husbandry, while the other bees could be kept alive for an extended time and this was particularly apparent for female bees. By delaying the start of the test to avoid inclusion of individuals from the cohort subject to early mortality, a greatly increased survival of control bees could be achieved.
- In addition to measuring survival of the adult bees in each test system, it was also possible to qualitatively score behavioural effects of the chemical on a range of characteristics including erratic movement, shaking, lethargy and failure to respond to external stimuli. Initially these endpoint were scored in a binary fashion (not impaired, impaired), but with suitable benchmarking a more comprehensive set of traits could be defined and scored.
- Tests with *A. mellifera* larvae were successful following an adaptation of the current OECD toxicity test guideline for this life-stage with high survival of unexposed control larvae. Both mortality and larval weight could be used as quantitative endpoints for the study.

4.2. Phase 2 - Toxicity of seven test chemicals for the honeybee *Apis mellifera* including analysis using DEBtox

- The chemicals selected for study cover representative chemicals from three insecticidal groups, neonicotinoids, pyrethroids and organophosphates, trace elements (cadmium and arsenic), a herbicide and a fungicide. The timing of the work was also re-planned between CEH and EFSA to take account of the specific requirements of the project, such as the need to link the acute and chronic tests to minimise animal use, and also the seasonality of bee species availability.
- The use of toxicity tests to provide an extended exposure of *A. mellifera* to each of the seven test chemical allowed datasets to be generated that examine the effects of exposure time on toxicity (expressed as metric such as the LC_{50}) through determining effect concentrations for selected test time-points.
- Data on survival in time was well suited to the parameterisation of DEBtox models for each chemical that could be used to estimate a series of key toxicological parameters relating to

inherent species sensitivity (the NEC, toxicokinetics (the elimination rate) and toxicodynamics (the killing rate)). The parameters derived from DEBtox model fits could be further used to predict the pattern of toxicity and from this LC_{50} values not only for relevant time-scale in the experiment, but also for biological relevant exposure times beyond test duration, such as the 720 h average summer life-cycle of a worker bee.

- Two tested chemicals, namely 2,4-D and tau-fluvalinate, did not show effects on survival at concentrations up to the water solubility and propiconazole showed only a partial mortality over 10 days at these levels. For the remaining four chemicals clear concentration and time dependent effects on survival were found. The observed time course of toxic effects could generally be modelled in detail with the DEBtox model.
- No Effect Concentrations (NEC) showed *A. mellifera* were most sensitive to the insecticides (clothianidin > dimethoate > tau-fluvalinate), followed by the trace metals and then fungicide and herbicide.
- Colony effects were found that may point to differences in sensitivity up to one order of magnitude between hives for two chemicals, clothianidin and arsenic. The relative small number of bee used from each hive for testing (10 per replicate), mean that the hive specific DEBtox models are relatively weak and further worker is therefore warranted to further investigate such variations.
- Trends for LC_{50} over time calculated from the DEBtox model fits indicated a 25 fold change when exposure is extended from 96 to 720 h for cadmium, the most time dependent chemical. This change was in the order of 10 fold, and < 10 fold for clothianidin and dimethoate. Hence to when comparing the effects of longer term exposure (e.g. 240 h and loner) with those from short-term test (e.g. 48 h or 96 h) it would be expected that calculated LC_{50} from the test of different duration would largely fall within a factor of 10 difference, except for chemicals with notably slow toxicokinetics such as cadmium.

4.3. Phase 3 – Comparative toxicity of the seven test chemicals for the three bee species

- Toxicity tests could be completed for all chemicals in each of the three selected species. Four of the selected chemical elicited a clear effect on survival in all three species (clothianidin, dimethoate, Cd, As). For the remaining three chemicals, mortality was not found in one or more of the tested species even when exposure was to the maximum water soluble concentration. This absence of concentration response was observed for tau-fluvalinate in *A. mellifera*, propiconazole in *A. mellifera* and *O. bicornis* and for 2,4-D in all three species. The concentration response curves for those chemicals showing overt toxicity and the LC_{50} values derived were generally in good agreement with the results of previous published studies.
- Measurement of feeding and knowledge of the body weights of the three species at the life-stages used for testing could be used to express exposure as a number of metric, namely concentration in sucrose solution food, dose consumed per bee and dose consumed per mg bee. Since bees are feeding continuously over the study, both measures of dose will change in time. Since DEBtox model fits used the data from all doses and time points the only time invariant metric of exposure that can be used is concentrations. When toxicity metrics are calculated for a single time-point exposure, a received dose can be used (and corrected for body weight). As a product of concentration and volume consumed, metabolism or excretion is not considered in this assessment. These processes may act to detoxify or remove the assimilated chemical. This means that although consumed dose can be estimated this is not likely to relate directly to the internalised concentrations. Hence, effect concentrations calculated based on such value may give a false impression of inherent sensitivity. As such they do not provide a clear interpretation of sensitivity, which in the continuous exposure system is more simply expressed by concentration. A metric that can also be easily related to measured environmental values.

- The ranking of the comparative toxicity of the test chemicals was broadly similar for each of the three selected bee species. Toxicity decreased in the order clothianidin > dimethoate > cadmium > arsenic > tau-fluvalinate > propiconazole >= 2,4-D. This ranking order is in agreement with what is known about the toxicokinetics and toxicodynamics of the selected chemicals for bees.
- For all chemicals where effects on survival were seen, the magnitude of this toxicity was increased with exposure time. This pattern of change was broadly consistent with that observed for *A. mellifera*. LD_{50} values generally decreased between the 48 h and 240 hr exposure time by a factor of at least 5. The temporal patterns of toxicity seen are amenable to analysis using the DEBtox model framework giving the potential to identify toxicokinetic and toxicodynamic parameters, as well as no effect concentrations.
- Comparisons between species indicated generally comparable toxicity for the chemical tested. The only tested chemical for which a degree of divergence was seen in the estimates of toxicity was for tau-fluvalinate. This chemical showed a higher toxicity to *B. terrestris* and *O. bicornis* than for *A. mellifera*. Tau-fluvalinate has a relatively low toxicity to bees compared to other pyrethroids primarily because it has a high rate of metabolism. The effects seen for *B. terrestris* and *O. bicornis* suggests that such metabolism may be slower in these two species than in the honeybee in cases where they are exposed to this pyrethroid.
- Behavioural traits are tractable to measure in toxicity test in association with measurement of survival. Measurement of sublethal effects indicated a higher sensitivity of behavioural changes to exposure. These differences were greatest for *B. terrestris* followed by *O. bicornis* and lastly *A. mellifera*. The increased apparent sub-lethal sensitivity compared to survival effects in *B. terrestris* may be related to an absence of tropholaxis and an effect of movement of feeding and further exposure. In *O. bicornis*, the capacity to measure more subtle behaviour effects may also contribute.
- Larval studies provide a further tool to assess toxicity. In these studies effect on both survival and final body weight could be measured in a relatively high throughput system. Studies identified only a relatively low minimal of the two insecticide even at quite high exposure concentrations. A clear effect was seen at high cadmium concentrations that are likely to be environmentally unrealistic. No potentiation of clothianidin or dimethoate toxicity by propiconazole was indicated.

4.4. Phase 4 – Mixture toxicity experiments

- With high quality single chemical toxicity data available, it was possible to design experiments to include treatments that were anticipated to cause both limited and pronounced toxicity effects for each toxic chemical included in the mixture study. This is important as it provides a basis against which to detect potentiation, alleviation, synergistic and antagonistic interactions. Such effects can be examined through the use of conventional concentration response modelling and the comparison of point based effect estimates. Dose response fits, coupled with MIXTOX and DEBtox models are suitable tools for this extended data analysis.
- For all chemicals where effects on survival were seen, the magnitude of this toxicity was increased with exposure time. Effects on mortality generally increased from the 48 h time interval until 240 hr exposure time. This patterns of effects with both concentration and time were general consistent with the results of the previous single chemical studies. The temporal patterns of toxicity seen are amenable to analysis using the DEBtox model framework giving the potential to identify toxicokinetic and toxicodynamic parameters, as well as no effect concentrations.
- The potentiation studies were generally successful in achieving a clear concentration related bee mortality response to the single chemicals in the absence of any observed mortality effect from the potentiating substances. The exception was in the study of tau-fluvalinate

potentiation of clothianidin effect in *Bombus terrestris*. In this case a direct effect of the second chemical, tau-fluvalinate, was found at both tested levels of this chemical. The fact, however, that two tau-fluvalinate treatments were included in this study has the advantage that it makes the data amenable to joint concentration effect fitting using the MIXTOX model. As such we anticipate being able to complete the joint effect analysis for this experiment.

- Comparisons of effect concentrations and the use for the default mixture toxicity model of CA and IA provide a good description of the observed joint effect data. Analysis for the majority of mixtures indicated that effects are additive. Dose response fits conducted in the presence or absence of a synergistic show no systematic difference for exposure of dimethoate with propiconazole and clothianidin with tau-fluvalinate. Clothianidin and cadmium and cadmium and arsenic mixtures are also additive according the mechanistic interpretation of IA.
- Initial observation of the clothianidin and propiconazole mixture suggested there may be a limited degree of interaction of the chemicals in our chosen binary mixtures. For the potentiating studies, the assessment with clothianidin and propiconazole pointed to a slight increased toxicity for the neonicotinoid in the presence of the fungicide. This effect was a maximum of approximately two fold in *O. bicornis*, but was almost absent in *A. mellifera*. The magnitude of such a change do not mirror the orders of magnitude changes that have been previously reported for studies between tau-fluvalinate and sterol biosynthesis inhibiting fungicides (Johnson et al., 2013). The absence of such an effect may reflect differences in the nature of metabolism of different insecticide groups between bee species.
- The 5 mixture toxicity studies conducted also point to a likely limited degree of interaction between the chemicals with the results predominantly being response patterns that may be related to additivity. Only in the case of the study with dimethoate and clothianidin in *B. terrestris* and for males, although not females or the combined data-set in *O. bicornis* is there any substantive evidence of interaction. This appears to be an antagonistic in those cases where it is seen, which allows the survival of this species in the presence of clothianidin at dimethoate concentrations that cause greater only for the exposure in which the neonicotinoid is not present. This interaction is also weakly supported by the DEBtox models which show possible interaction, most clearly in the fit for *B. terrestris* and also for *A. mellifera*.

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Glossary

Concentration addition	A chemical mixture effect prediction model for chemicals with a similar mechanism of action, which is based on the dilution principal that joint effects can be predicted as the sum of toxic units when toxic units derived from the exposure concentration over the known LC_{50} for the chemical.
Independent Action	A chemical mixture effect prediction model for chemicals with a different mechanism of action, which is based on calculation of the product of unaffected fraction to predict joint effect.
Dynamic Energy Budget	A theory based on first principles where general rules for metabolic organisation are derived and used to understand how resources are used by organisms to support a range key biological traits.
DEBtox	A theory based on first principles where general rules for metabolic organisation are derived and used to understand how resources are used by organisms to support a range key biological traits.
MIXTOX	A statistical model used to find (concentration dependent) deviations from Concentration addition and Independent action models for binary mixture toxicity datasets, which are interpreted as interactions.
No Effect Concentration	The highest concentration having no effect on the test organism over extended (infinite) exposure time.

Abbreviations

EFSA	European Food Safety Authority
CA	Concentration addition
IA	Independent action
NEC	No effect concentration
LCx	Lethal concentration for x% of species
ECx	Effect concentration for x% of species
2,4-D	2,4-Dichlorophenoxyacetic acid
OECD	Organisation for Economic Cooperation and Development

Appendix A – Test methods used for *Apis mellifera* adults

Tests for all three of the selected bee species were established in the first year of the project each optimised in turn for issues of husbandry, effective dosing and the measurement of effects on the bees. The unique habits of the three species naturally required the development of different solutions to these issues in each case. The first set of single chemical studies showed that the developed tests were differently optimised for the three species. For *Apis mellifera* and *Bombus terrestris*, the test provided a robust, reliable and repeatable method to prolonged exposure via feeding and the measurement of progressive effects of exposure on survival over a 10 day period. For that reason, the same assay design used for the single chemical assessments in 2014 was used for all testing for the second stage analysis of mixture effects. For the solitary bee *Osmia bicornis*, initial trials with the single chemicals indicated that prolonged oral exposure assays were feasible for the species. However, control mortality rates were generally higher that would be desirable for a robust toxicity tests for this species in the first set of experiment. As a results in the second season a further set of optimisation studies were conducted with this species to improve the survival of the bees over the full 10 day test durations. This work was intended to provide an enhanced assay that would be better able to identify complex mixture effects. Details of the test bioassay used are set-out below.

General considerations: There are well established protocols for the oral and contact acute toxicity testing of chemicals including pesticides and other contaminants for adults (OECD 1998, 1998) and oral acute single exposure for larvae (OECD 237, 2013) of *A. mellifera*. More recently, OECD released a draft guidance document for repeated exposure for larvae (OECD, 2014). All exposures were designed to provide an exposure situation that simulated an extended (240 h) continuous intake via diet, since major current concerns relate to the interactions with systemically applied neonicotinoid insecticides for which there is known exposure via nectar, pollen and plant guttation water with other chemicals. Indeed, the EFSA Scientific Opinion on the risk assessment of plant protection products on bees (EFSA PPR Panel 2012) indicates that nectar foragers are potentially the most exposed category of honeybees via oral exposure. All tests used design aspects taken from established OECD protocols for oral exposure. However, for each species specific modifications to available standard procedures were used to allow both for the prolonged exposure and also for an increased observation frequency for effects on survival to support DEBtox parameterisation and also sub-lethal (behavioural) response assessment.

Husbandry: Colonies of *A. mellifera* were obtained as nucleus hives, each with a new queen in April 2014 from a local beekeeper. A total of 8 standard hives were populated with the bees from these nucleus hives and maintained in an outside apiary at the CEH Wallingford site. Hives were sited to be South facing and surrounded by hedgerows. During 2014, bees were maintained according to standard local bee keeping practice. This routine involved regular hive inspections every week to 10 days during the period April-September. These standardised inspections ensured that the colony was queen right and there was healthy brood/adult bees. The presence of pests and pathogens was also assessed. There was low level *Varroa destructor* noted in toward the end of both the 2014 and 2015 seasons and in 2015 one hive showed signs of *Galleria melonella*. Neither warranted any treatment as both were at a low level but were excluded from further experimental tests as a precaution.

After the receipt of colonies in April 2014, swarm capture in the following summer increased the number of hives to 11. At the end of the summer season in 2014, all 11 hives were prepared for overwintering (see below). After 2014experiments were complete, every colony was prophylactically treated with Thymol crystals for control of *Varroa destructor* mites. In March 2015, the overwintering hives were reopened and checked and the Thymol removed. This was at least two months before any adults or larvae were used in the 2015 experiments. After overwintering, three hives were found to have been lost, with these hives each lacking a queen and having a greatly reduced number of workers. At this time, each colony was provided with fondant to ensure they were not stressed

through resource limitation early in the season. Two of the hives were re-Queened during 2014 and these were not used for experiments in 2015. All other colonies were Queen right, appeared healthy with no visible pathogen or mite presence and had overwintered successfully.

Toxicity test procedure. For each test, even aged, adult worker honeybees were collected from frames containing young brood. The number of replicates hives used for each test treatment depended on the design of the specific experiment (due to the different treatment number used). Four replicate hives were used for each treatment for the single chemical studies in 2014 and three per treatment for the mixture tests conducted in 2015. Each test replicate comprised groups of 10 bees from a single hive. The same test bioassay cages were used for all tests. Within the container, food was supplied as a sucrose solution (50% w/v) in purified milli-Q distilled water from a feeder, inserted into the test cage.

On the day of the test, young worker bees were harvested from 1-2 frames directly from within each of the 3 replicate test hives. Each frame was gently shaken out into a container that was then immediately covered. Bees were immediately transferred to an outside insectary facility where, when needed for replicate set-up, they were initially chilled at -20ºC for no longer than 45 s to anaesthetise the insects. This 45 s period was found to be optimal to slow bee activity to the extent that it allowed them to be individually picked up with soft forceps without them either clustering together excessively, or alternatively being subject to mortality. Bees were loaded individually into bioassay chambers. Once all the bees from a replicate hive had been loaded into chambers, syringes loaded with test chemicals or control sucrose (+/- acetone) were placed into the cage in a feeder. Each test unit was placed in a controlled environment room at 25ºC ± 2°C in the dark for the full 10 days duration of the experiment.

The assessment of chronic toxicity was conducted by extending the initial acute tests from 96 to 240 h. The approach is in agreement with the recommendations from the EFSA Scientific Opinion on bees (EFSA PPR Panel, 2012) and the Tender requirement to minimise the number of bees tested. Throughout the extended exposure, mortality of the incubated colony associated test group (10 individuals from each of three separate hives) was recorded daily, with assessment of number of dead bees at each of the 19 time points. This monitoring schedule is an important aspect of the project because it is vital for the production of datasets for single chemicals and mixtures using the method based on Haber's law as described by the EFSA PPR Panel (EFSA PPR Panel, 2012) and also importantly for DEBtox analysis.

The exact experimental design used depended on the nature of the test to be conducted. Single chemical toxicity studies involved exposure of the replicate hive to each of a series of concentrations of the test chemical in the sucrose food source. Between 5–7 different test concentrations were tested. Mixture studies were of two types designed to assess potentiation or joint toxicity. Potentiation designs involved exposure of bees to a concentration series of one chemical at both toxic and non-toxic concentration in the presence of a second chemical predicted to have no effect on mortality at the two exposure levels used. The mixture toxicity tests in contrast included multiple single chemical and mixture effects designed from 96 h LC_{50} values calculated from the single chemical studies (see Section 1.1 for full design details). For chemicals that needed to be made up initially in acetone, dilution was made to give a final concentration of 1% acetone in the test chemical. In those cases where both of the two chemicals required preparation in acetone, the tests were designed in such a way that each did not contribute more that 0.5% acetone into the final dosed sucrose solution. Negative controls used were in all cases including sucrose alone and in cases where acetone was used for dosing also a sucrose solution containing 1% acetone. In addition, 4 replicate positive of a control

were run, this being with a concentration of dimethoate calculated at the 96 h LD_{50} from initial single chemical trials in those tests where dimethoate itself was not included among the tested chemicals.

Exposure was by providing bees with access to sucrose solution in distilled water (with a final concentration of 500 g/l, 50% w/v) spiked with the test chemicals (dissolved in either acetone or water) at the required test concentration. For each test conducted, test groups of bees were randomly allocated to test cages and for each single chemical exposure. During the preparation phase it was ensured that bees were without access to food for a maximum of 1–2 hours before initiation of the test. Chemicals were introduced via feeders and all control and treatment cages were randomly placed in a dedicated constant temperature facility (temperature = 25 ± 2°C; relative humidity ~ 60%; in the dark). The treated and untreated control sucrose solutions were administered to the bees *ad libitum* throughout the test. The test feeders were weighed at the start of the experiment (time zero) and then at 48 h, 96 h and 240 h to allow the calculation of food consumption up to each of these time points. Mortality was recorded three times daily during the first 96 hours of the exposure and then once a day up to 240 h when the experiment was ended. In the mixture tests, this was extended to also include the recording of aberrant behaviour (erratic movement, shaking, lethargy) for individuals. The results arising from each experiment include data on mortality at each measurement time-point, as well as observed behavioural effects. The mortality data are suitable for future fitting of standard statistical models such as probit analysis (linear regression of survival after probit transformation and log concentration) and a widely accepted statistical method in understanding dose-response relationships. Potentiation experiments also used a similar probit analysis approach to assess response variable (e.g. LC_{50}) for concentration series for the toxicity chemical in the absence and presence of the potentiating chemical and then comparing these between exposure. For the mixture toxicity studies, MIXTOX model fits in order to quantify any potentiation of alleviation of toxicity in the potentiation studies and fits of the observations compared to predictions of CA and IA in the mixture toxicity tests. Sub-lethal data could also be analysed using logistic function to derived parameter estimates including EC_{50} values to compare between species exposed to clothianidin. Additionally, the data on patterns of mortality in time could be used to support the derivation of appropriate toxicokinetic and toxicodynamic parameters using the DEBtox modelling approach of Baas et al. (2007).

Appendix B – Test methods used for *Apis mellifera* larvae

General considerations: The *A. mellifera* larval toxicity tests was based on a procedure initially developed by Aupinel et al. (2007), which has recently been standardised into a draft OECD guideline (OECD, 2012). The method enables determination of the lethal dose (72-h LD_{50}) following single exposure of honeybee larvae to chemicals for a define exposure period followed by subsequent survival monitoring. Methods for rearing and collection of the required test organisms followed an improved method from the procedure outlined in the Draft OECD test guidelines that significantly increased control larval survival during experiments following methods of Genersch et al. (2005a, b). Synchronised larvae at 3 days olds are used for the assay, with individuals placed into 24 well-plates where they have access to a diet comprising a mix of 66% royal jelly and 33% of an aqueous glucose : fructose solution.

Toxicity test procedure: Larvae of honeybees were maintained individually on a diet comprising 33% of a solution containing 9% fructose and 9% glucose and 66% of royal jelly (resulting food therefore contains 66% royal jelly : 3% glucose : 3% fructose). Solutions were prepared by mixing the two ingredients in batch preparation per treatment dose. Solutions were then kept at 35°C prior to use in a water bath. In cases where diet was dosed, the chemical was added to the fructose : glucose solution using acetone as a solvent carrier when necessary. For the start of each bioassay, 300 µl of appropriate control and spike diets were dispensed into 24 multi-well cell culture plates ready for the addition of an individual larva to each well. Aliquots were added to each well using a positive pressure syringe and spread on the bottom of the well under aseptic conditions. The experiment was conducted over a total period of 4 days with exposures made on the second day followed by a 72 h period for observation of effects on survival and larval growth rate.

One Day 1, 1 day old larvae from frames from within the selected replicate hives were transferred in groups of 10 to individual cells in cultures plates containing 300 µL freshly prepared clean and unspiked diet. A grafting tool was used to lift individual larvae out of the brood frame and transfer them to diet, ensuring that the same orientation of the larva was maintained such that the spiracles were upright, thereby preventing suffocation and ensuring successful feeding. Each plate had 6 wells loaded only with sterile water to ensure humidity within the plate was maintained. All plates were warmed to 35°C prior to loading larvae and once loaded, were kept a 35°C in the dark in an incubator with no fan circulation and under high humidity (trays of water placed in the bottom of the incubator). After collection from frames, the larvae were held on this fresh diet for an initial period of 24 h to ensure any handling deaths were eliminated, prior to exposure. After 24 h, larvae were transferred to new 24 well plates containing either 300 µL of diet spiked with chemicals at the required exposure concentration or un-spiked control diet and incubated for a further 24 h. Concentrations required to achieve specific doses were based on an assumed consumption of 30 µl of the diet per individual larvae. After 24 h incubation on the spiked diet, larvae were checked and any mortality recorded (dead larvae are opaque, flattened and shown no movement or feeding when examined under a binocular microscope). Larvae were transferred at 24 h to un-spiked diet and further checked at 48 h and 72h for mortality. At 72 h, s surviving larvae were weighed to assess sub-lethal effects.

Appendix C – Test methods used for *Bombus terrestris* adults

General considerations: The chemical toxicity test regimes for other species of euscocial bees are less well established and are not captured by standard OECD protocols. However, the use of queenless microcolonies for bumblebees has been established and used for a range of studies including assessing the toxic effects of GM crops (Mommaerts et al. 2011; Laycock et al. 2012; Laycock et al. 2014). The basic method has been described by Regali and Rasmont (1995) and Tasei et al. (2000) and is also specifically recommended by EFSA PPR Panel (2012). One issue that was raised by EFSA PPR Panel (2012), was whether there was a need for bumblebees to be fed individually because of lack of trophallaxis. This was because variation in feeding rate within individuals in microcolonies could result in differences in individual exposure, leading to greater experimental variation. Individual feeding in our tests would, however, mean using a different design from the microcolony approach and move the focus from the effect at colony level to individuals in an exposure system that would not reflect bumblebee colonial habit. Therefore, it was agreed to proceed with the microcolonies approach. This design was successfully used in 2014 for the single chemical studies and was mirrored in 2015 for mixture tests.

Toxicity test procedure: Native *Bombus terrestris audax* were obtained as commercially reared colonies with 30–40 workers from NV Biobest, Belgium. When colonies arrived, they were immediately switched from 'Biogluc' to be maintained on 50% w/v sucrose and supplemented with fresh pollen. A total of 11 colonies were used as a source of experimental adult bees. Microcolonies were established to comprise three workers selected at random from a single colony. The same bioassay container design used for honeybee trials was used for the honeybee toxicity tests. For each assay, bees were taken from a minimum of 4 colonies and a maximum of 10. On the day of the test, young adult worker bees of a similar size were removed with long forceps from colonies and loaded into containers as for the honeybee tests. Individuals were not anaesthetised in any way, as adults could be relatively easily caught and handled meaning that no additional intervention (e.g. chilling) was required prior to loading.

The replicate test microcolonies were randomly allocated to treatments with a minimum of 3 replicates for each exposure treatment, each from different source colonies. This included a standard dimethoate positive control at the 96 h LD_{50} concentration and a negative sucrose only and +/- acetone control as appropriate for the range of chemicals used in a particular study. Within the container, single and binary mixture chemical concentrations and non-treated sucrose solutions were supplied using a feeder as for the honeybees. The exposed microcolonies were maintained in a dedicated constant temperature facility at 25 ± 2°C, ~ 60% RH, in the dark. Adult mortality was recorded three times daily during the first 96 hours of the exposure to allow an initial assessment of acute toxicity. Thereafter, survival was monitored at 24 hour intervals (up to 240 hours) to capture effects of chronic cumulative exposure. At each time-point (Time points used were, thus, 0, 3, 19, 24, 27, 43, 48, 51, 67, 72, 75, 91, 96, 120, 144, 168, 192, 216, 240), bees were scored also for behavioural signs of overt toxicity, counting both bees showing 'normal' and those showing aberrant behaviour indicated by erratic movement, shaking and lethargy.

Appendix D – Test methods used for *Osmia bicornis* adults

General considerations: The solitary bee species selected for toxicity testing *Osmia bicornis* is native to the UK and is known to be an important pollinator of plants, including commercially important crops. *O. bicornis* has a physiology and ecology that is typical of solitary bee species within the *Osmia* genus. The species is commercially available as field collected bee pupae obtained from nesting structures. The suppliers of *O. bicornis* pupae identified a number of issues with the supply and maintenance of this species in the laboratory that could have impacted the experimental testing program. One issue was the relatively high level of parasitism and pathogens that could be expected to occur among the supplies stock. Estimates of pupal parasitism indicated prior to the start of the testing work that may range from 20–60%. This factor had the potential to greatly reduce the available commercial supply need for testing. To address this 3 x the required number of pupae that would be needed to produce the necessary bees for testing were ordered in each experimental season. An additional risk to organism quality comes due to the presence of pathogens (fungal, bacterial, viral) for which infection rates of wild collected pupae are poorly described in this species. These pathogens might also have the potential to affect the sensitivity of the solitary species to the selected test chemicals, with a further possible impact on the experiments being the potential for higher control mortality than might be expected in the well maintained honeybee hives that are used for standard toxicity testing. A further issue for testing was the possible challenge in getting *O. bicornis* to reliably feed in the laboratory.

There has been only limited use of *Osmia* spp. in toxicity testing (Ladurner et al., 2003; Ladurner et al., 2005) although this work has included the initial development of protocols specifically for *O. bicornis* (Tesoriero et al., 2003; Konrad et al., 2008). These studies, and initial trials conducted in early 2014 provided the basis for the design of an appropriate testing protocol during the project. In early 2014, a series of trials were conducted to support the refinement of the overall bioassay system design, container types, test chemical feeding methods and recording systems. These studies have provided the basis for the design of an appropriate testing protocol. The initial test of this procedure was to assess the toxicity of the seven single chemical. This allowed an assessment of the effectiveness of the test procedure across multiple bioassays. These initial set of bioassays confirm the suitability of key aspects of the test, such as the approach to pupae rearing and hatching, the cage structure and the design of the feeder units. A specific problem with high control mortality (20–50% over a 240 h exposure) was, however, encountered. Initial work conducted in the second season, was specifically designed to address this issue. The aim of the work was to provide approach to the initial rearing and selection of hatch bees that would increase control mortality too levels close to those required in the OECD adult honeybee test procedure.

Toxicity test procedure: Overwintered *O. bicornis* pupae were received in March 2015 from a German stock. These were immediately stored at 4 ± 1°C, 65 ± 10% RH with no light. Pupae remained viable with minimum adult emergence as long as this low temperature was maintained. On receipt, bees were size segregated, with the large pupae corresponding to females and the smaller pupae males. Previous work in 2014 identified that pupae weight < 0.11 g generally corresponded to males whilst those > 0.11 g were females. Whilst this relationship was not so precise in 2015, these approximate weights allowed us to segregate pupae relatively accurately. Warming of the required number of pupae (usually 150% of the number required for each test) at 28°C allowed bees to emerge at 1–4 days incubation. At the beginning of the experiments (March), males were warmed for 2 days but by May only required 24 h warming. Similarly, female pupae required 3 days early in the year but this reduced to 2 days later in the year. Emergence success for the received cohort always remained in the range of 80% hence, there was no suggestion that storage had a strong effect on

viability. Even so we made sure to complete all tests by June to avoid using pupae stored for longer than the normal emergence period of the species in experiments.

Within the test container, food was supplied as a sucrose solution (20% v/v sucrose (Sigma; high grade for molecular biology) in purified distilled water that was loaded into plastic 5 ml in which the tip had been cut off to provide a drinking hole of approximately 2 mm. Test solutions were prepared in water or acetone prior to experiment start and then stored ready to mix with sucrose solution. For all experiments, 10 individual bees (5 males, 5 females) were exposed to each concentration in individual pots.

Once feeders had been loaded and supplied to the adults, all bees were kept in a controlled temperature glasshouse at 22 ± 2°C, ~ 60% RH, under natural photoperiod. In pre-trials, this housing was found in to work better than an indoor constant temperature room under artificial light, supporting increased feeding and a clear diurnal behaviour pattern. Over the course of the experiment, continuous monitoring of adults allowed patterns of survival to be assessed. Additionally aberrant behaviour was assessed to indicate sub-lethal effects on characteristic relating to movement or metabolism. Mortality was recorded three times daily during the first 96 hours of the exposure to allow assessment of acute toxicity. Thereafter survival was monitored at 24 hour intervals (up to 240 hours) to assess the effects of chronic cumulative exposure. Time points used were, thus, 0, 3, 19, 24, 27, 43, 48, 51, 67, 72, 75, 91, 96, 120, 144, 168, 192, 216, 240 h. Recording of normal and aberrant behaviours were also made on an individual basis as each time interval by recording the number of bee showing aberrant movement behaviour and lethargy. Any effects relating to the exposure (trembling, erratic flying, etc.) were also recorded as either absent (i.e. normal behaviour'), moderate or severe.

Test optimisation: Experiments in 2014 indicated that it is feasible to emerge *Osmia bicornis,* to dose bees by providing access to concentration of a test chemical dissolved in a sucrose solution and to maintain exposed bees for an extended 10 day period. The test design used bees commercially supplied as field collected pupae obtained from outdoor maintained nesting structures. Contacts with suppliers collecting *O. bicornis* from managed wild populations identified a number of possible issues with the supply and maintenance of this species that could impact on our experimental testing protocols. One possible problem identified was the relatively high level of parasitism and pathogens that could be expected to occur among the supplied stock. Measures of actual rates of emergence failure indicated that the supplied population had a viability of around 80% which is towards the top end of the expected range. Hence, suitable bees for test could be readily obtained from stock without the need for hatching of a large contingency of pupae.

Once emergence had taken place, a problem that was found in the 2014 single chemical studies was the relatively high rates of control mortality. This rate was in the region of 25–50% across the seven tests conducted. It was also higher in females than males. A review of practices during single chemical tests, suggested that one factor that may be initially compromising the solitary bees was that they were emerged individually without immediate access to food. To test for this, a detailed study was run to assess if different hatching and feeding options could allow improve the background health of individuals included in our tests. These tests trialled different options for feeding after emergence (no feeding vs immediate feeding) and the use of sucrose diet of different concentrations (20% vs 50%). Based on the outcomes of this test which are described in detail in results section, the best approach to initial husbandry and rearing was integrated into our basic test bioassay design and then used for the mixture bioassays with this species.

Appendix E – Data analysis approaches including DEBtox modelling

Single chemical and potentiation experiments: all single chemicals experiments and also the each concentration series of the toxic chemicals (i.e. those in the absence and presence of concentrations of the second chemical) in the potentiation experiments were designed to include a full concentration response series of six treatments, including appropriate controls. In the potentiation experiment, a probit analysis was conducted for each concentration series based on an assumption of no contribution of the potentiating chemical to the toxicity of the toxic chemical. Hence in the absence of interaction between chemicals, similar toxicity metric would be expected to be derived for the toxicant in the tests conducted both in the absence of and presence of the second chemical.

Mortality data for the different single chemical series was analysed using a probit method. Probit analysis is a type of regression used to analyze binomial response variables. The technique transforms the sigmoid dose-response curve to a straight line that can then be analyzed by regression either through least squares fitting. Probit is an established model for data analysis for survival data for bioassays with pesticides. Indeed it was originally specifically designed for this purpose. Probit analysis is also the recommended approach for the analysis of toxicity data collected from toxicity tests for honeybees conducted according to OECD standardised test procedures. From the fitted models for each single chemical effect series, the LC_{50} concentrations for the effects of the tested chemical on the specific species studies for the specific time-point being considered can be estimated. Additionally the model can be used to derive estimates for lower effect concentrations. However as the desired effect level approaches either high or low effect levels, the reliability of the estimates is greatly reduced meaning that such values should be treated with extreme caution. The use of DEBtox parameters provides and alternative way to derive these low effect concentrations estimates. The DEBtox based approach has the further advantage that it is more robust because the estimates of effect concentrations are made using parameters derived from the data from all time points.

In the tests conducted in 2015, data for effects of the single chemicals on behavioural traits, as well as survival, was also collected. This data was also binary in nature representing the number of normal and behaviourally impaired bees. The purpose in collecting this behavioural data was three-fold. Firstly to assess the feasibility of including assessment of behavioural effects as routine measurement parameters within tests with each of the three selected species; secondly to assess comparative sensitivity of behaviour traits in each species compared to morality effects; thirdly to compare the sensitivity of behavioural traits to the same chemical between species. To allow assessment in respect of each of these objectives, the data collected in tests clothianidin alone during potentiation experiments conducted in 2015 has been analysed in details for all three bee species. Analysis of this data included tracking behavioural effects in time and also estimation of effect concentrations ($EC_{50,behaviour}$) from behavioural data to compare between species and also to LC_{50} values. $EC_{50,behaviour}$ values were estimated by fitting probit models to the binomial response variables used (non-impaired, impaired).

Data analysis for the mixture toxicity experiments: the analysis of the mixture toxicity experiments utilised a paradigm of predicting/estimating the joint effect of multiple non-interacting chemicals through 'addition' has been developed and tested based on the two underpinning concepts of CA and independent action (Van Gestel et al., 2010). If chemicals have the same mode of action, their combined toxicities can be described by the CA model, according to:

$$\sum_{i=1}^{n} \frac{c_i}{\mathrm{EC}x_i} = 1 \qquad \text{(Eqn. 1)}$$

Where c_i gives the concentration (or dose) of the i-th component in an n-compound mixture which elicits x% total effect and ECx_i denotes the concentration of that substance which provokes x% effect if applied singly.

Every fraction c_i/ECx_i - also termed a 'toxic unit' - gives the concentration of a compound in the mixture scaled for its relative potency. If the sum of the toxic units for all mixture equals 1, then CA holds.

Alternative if two stressors (chemicals or chemical / non chemical) have different modes of action then their combined effects can be described by the independent action model, according to:

$$Y = u_0 \prod_{i=1}^{n} q_i(c_i) \qquad \text{(Eqn. 2)}$$

Where Y is the measured biological response, u_0 denotes either the control response for endpoints that decrease with increasing dose (e.g. survival or reproduction) or the maximum response for endpoints the increase with increasing dose (e.g. mortality or enzyme induction), and $q(c_i)$ denotes the probability of non-response (i.e. the unaffected fraction), functionally related to concentration c of compound i.

By accepting CA and IA as suitable reference models, we are then able to use these models as the default predictions against which to identify the frequency of cases where they fail to describe an observed joint effect. To assess the fit of observed effect against CA and IA model predictions, we analysed the data using the MIXTOX model of Jonker et al (2005). This descriptive (rather than predictive) model offers a data analysis framework that enables the detection and quantification of significant deviations from either the CA or IA models. To do this for CA, the fact that the sum of toxic units (Equation 1) of all chemicals should equal 1 where CA describes the data can be rewritten as follows for binary mixtures:

$$\frac{c_1}{f_1^{-1}(Y)} + \frac{c_2}{f_{f2}^{-1}(Y)} = \exp(G) \qquad \text{(Eqn. 3)}$$

Where c_1 and c_2 denote the concentrations of the individual chemicals in the mixture, Y indicates the measured biological response, f_1^{-1} and f_2^{-2} indicate the inverse dose response functions (inverse of Equation 2, so dependent on a joint 'max' and individual EC_{50} and β values) for the individual compounds in the mixture and G denotes an excess function to quantify deviations from the CA model (Jonker et al., 2005).

The procedure for modelling the data of an experiment of the design used here following CA principles or IA principals relevant to the different model, relies on finding the values of the joint 'max' and the individual EC_{50} and β values that best allows the description of all data points in the single compound response curves and the full mixture response surface where G is zero (for details see Jonker et al., 2005). In cases where CA describes the data fully the value of 'exp(G)' should be 1, hence the value of the 'deviation function' G would be zero. This parameter fitting was done by considering Equation 3 for all data points simultaneously while minimising the sum of the squared residuals (*SS*).

To assess if the CA model alone provided an adequate description of the data, or there were systematic deviations where more or less severe effects than should be anticipated from CA predictions, a stepwise approach was used. In this, extra parameters that could describe biologically meaningful interactions between the two chemicals were sequentially added to the deviation function G. The first parameter added was *a*. This describes either overall antagonistic (effect less than predicted by CA) or synergistic (effect greater than predicted by CA) effects. The second parameter added was either b_{DR} or b_{DL}. These respectively describe a ratio dependent effect relative to CA dependent on the proportional contribution of each stressor for b_{DR} and an effect level dependent response relative to CA dependent on the magnitude of the impacts observed at different exposure severities for b_{DL}.

Model with more parameters usually showed improved fits preventing direct comparison between models. The sequential parameter addition used here, however, creates a nested set of models which allows for testing the statistical significance of the improvement in fit from the extra parameters. This

significance testing is completed by using the resulting *SS* for pair wise model comparison through likelihood ratio testing at degrees of freedom equal to the difference in the number of parameters in the two models through Chi-squared (χ2) tests as described by Jonker et al. (2005). The only two models that are not nested are the ones for ratio- or effect level-dependence, hence these cannot be directly pair wise compared using this approach. Numerical values calculated for the deviation parameters can be interpreted for parameter values according Table 11.

Table 11: Interpretation of additional parameters substituted into the concentration addition (CA) reference model that define the functional form of the deviation pattern

Parameter	Value	Interpretational meaning
		Synergism/Antagonism
a	< 0	Synergism
	> 0	Antagonism
		Ratio dependence
a	< 0	Synergism, except for those mixture ratios where significant positive *bi* indicate antagonism
	> 0	Antagonism, except for those mixture ratios where significant negative *bi* indicate synergism
b_i	> 0	Antagonism where the toxicity of the mixture is caused mainly by toxicant *i*
	< 0	Synergism where the toxicity of the mixture is caused mainly by toxicant *i*
		Dose level dependence
a	< 0	Synergism at low concentrations and antagonism at high
	> 0	Antagonism low concentrations and synergism at high
b_{DL}	> 1	Change at concentrations lower than the EC_{50}
	= 1	Change at the EC_{50} concentration level
	$0 < b_{DL} < 1$	Change at concentration levels higher than the EC_{50}

Data analysis for single chemicals and mixture using DEBtox: data on mortality for each of the three species provided time series data that was suitable for modelling of the pattern of effects using the DEBtox model. DEBtox is a biologically-based model initially developed by Kooijman and Bedaux (1996), that is based on the DEB theory. The approach we chose is based on mechanistic model for survival compatible with the principals of DEB theory. This took the form of a scaled one-compartment model to describe uptake and elimination and a hazard model to describe survival. This model needs four time-independent parameters to describe the whole time course of the toxic effect:

- The Blank Killing Rate, which is a measure of the rate of background mortality in a population not subject to any chemical exposure (hr^{-1}).
- The No Effect Concentration (*NEC)*, a time-independent toxicological threshold below which no effects occur even after life-long exposure, it is expressed as an environmental concentration in mmol/L.
- The killing rate (k_k), the toxic potency of the compound (once the NEC is exceeded) expressed in $(mmol/L)^{-1}$ d^{-1}.
- The elimination rate (k_e), which describes when the equilibrium between internal and external concentration is set, expressed in d^{-1}.

This model was originally developed by Kooijman and Bedaux (1996) and developed by Jager et al. (2011), who also provided a conceptual comparison of the different survival models that are currently used, the underlying assumptions and how the different models are related. In the context of the long-term risk assessment for the effects of chemical exposure on cohort population of the three tested bee species, the NEC is particularly important as this parameter represents the concentration expected to result in increased hazard that will be realised following long-term exposure. Whether

these effects will be fully observed will depend of the kinetic rates of uptake. For slowly accumulating chemicals, the full hazard may not be fully realised in a short-term laboratory test or even following life-time exposure. This is because the period of the life-span may not be of sufficient time for internal concentrations to reach equilibrium.

For the analysis of survival data, effects in mixtures, analysis with DEBtox can be applied following the framework initially proposed by Baas et al. (2007). Within this approach, the effects of exposure to two compounds are simultaneously analysed. For the exposure to two compounds simultaneously, the effects are described by the toxicity parameters of the individual compounds, extended with an interaction parameter. When model fit indicates no interaction parameter then the mixture is additive. When this is the case the long-term effects of each component in a mixture will be related directly to the attributes to the no effect concentration and the elimination rate and killing rate will be as for the single mixtures. If an interaction is found, then an additional parameters included in the model will provide a significantly improved fit of the model to observed effects in time over the whole dataset. This application of DEBtox for mixture to analyse survival effects in time, thus gives insights into the overall nature of synergistic or antagonistic effects and will also give toxicokinetic and toxicodynamic insights relevant to the case.