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Investigating combined toxicity of binary mixtures in bees: Meta-analysis of laboratory tests, modelling, mechanistic basis and implications for risk assessment

Edoardo Carnesecchi[a,b], Claus Svendsen[c], Stefano Lasagni[d], Audrey Grech[e], Nadia Quignot[f], Billy Amzal[f], Cosimo Toma[b], Simone Tosi[g], Agnes Rortais[h], Jose Cortinas-Abrahantes[h], Ettore Capri[i], Nynke Kramer[a], Emilio Benfenati[b], David Spurgeon[c], Gilles Guillot[j], Jean Lou Christian Michel Dorne[h,k,*]

[a] Institute for Risk Assessment Sciences (IRAS), Utrecht University, 3584 Utrecht, the Netherlands
[b] Laboratory of Environmental Chemistry and Toxicology, Department of Environmental Health, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, via Mario Negri, 2, 20156 Milano, Italy
[c] Centre for Ecology and Hydrology, Maclean Building, Benson Lane, Crowmarsh Gifford, Wallingford OX10 8BB, UK
[d] Arpae Emilia Romagna, Bologna, Italy
[e] INERIS, Paris, France
[f] CERTARA, Paris, France
[g] Epidemiology Unit, European Union Reference Laboratory (EURL) for Honeybee Health, University Paris Est, French Agency for Food, Environmental and Occupational Health and Safety, Paris, France
[h] European Food Safety Authority (EFSA), Scientific Committee and Emerging Risks Unit, Parma, Italy
[i] Università Cattolica del Sacro Cuore, Dipartimento di Scienze e Tecnologie Alimentari per una filiera agro-alimentare Sostenibile (DiSTAS), Piacenza, Italy
[j] International Prevention Research Institute, Lyon, France
[k] School of Biosciences and Phenome Centre Birmingham, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

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ABSTRACT

Bees are exposed to a wide range of multiple chemicals "chemical mixtures" from anthropogenic (e.g. plant protection products or veterinary products) or natural origin (e.g. mycotoxins, plant toxins). Quantifying the relative impact of multiple chemicals on bee health compared with other environmental stressors (e.g. varroa, viruses, and nutrition) has been identified as a priority to support the development of holistic risk assessment methods. Here, extensive literature searches and data collection of available laboratory studies on combined toxicity data for binary mixtures of pesticides and non-chemical stressors has been performed for honey bees (*Apis mellifera*), wild bees (*Bombus* spp.) and solitary bee species (*Osmia* spp.). From 957 screened publications, 14 publications provided 218 binary mixture toxicity data mostly for acute mortality (lethal dose: LD_{50}) after contact exposure (61%), with fewer studies reporting chronic oral toxicity (20%) and acute oral LC_{50} values (19%). From the data collection, available dose response data for 92 binary mixtures were modelled using a Toxic Unit (TU) approach and the MIXTOX modelling tool to test assumptions of combined toxicity i.e. concentration addition (CA), and interactions (i.e. synergism, antagonism). The magnitude of interactions was quantified as the Model Deviation Ratio (MDR). The CA model applied to 17% of cases while synergism and antagonism were observed for 72% (MDR > 1.25) and 11% (MDR < 0.83) respectively. Most synergistic effects (55%) were observed as interactions between sterol-biosynthesis-inhibiting (SBI) fungicides and insecticide/acaricide. The mechanisms behind such synergistic effects of binary mixtures in bees are known to involve direct cytochrome P450 (CYP) inhibition, resulting in an increase in internal dose and toxicity of the binary mixture. Moreover, bees are known to have the lowest number of CYP copies and other detoxification enzymes in the insect kingdom. In the light of these findings, occurrence of these binary mixtures in relevant crops (frequency and concentrations) would need to be investigated. Addressing this exposure dimension remains critical to characterise the likelihood and plausibility of such interactions to occur under field realistic conditions. Finally, data gaps and further work for the development of risk assessment methods to assess multiple stressors in bees including chemicals and non-chemical stressors in bees are discussed.

* Corresponding author.
E-mail address: jean-lou.dorne@efsa.europa.eu (J.L.C.M. Dorne).

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1. Introduction

Worldwide, bee species such as the honey bee (*Apis mellifera*), bumble bees (*Bombus* spp.) and solitary bees (e.g. *Osmia* spp.) are essential organisms for the environment particularly for their critical roles in the pollination of crops, flowers and fruit trees and consequently their economic impact (Kennedy et al., 2013; Burkle et al., 2013; Potts et al., 2010). In the world, 75% of food crops (e.g. cacao, almond, apple etc.) relies on animal-mediated pollination (Klein et al., 2007) and the majority of human micronutrients (e.g. vitamins, minerals) derive from pollinator-dependent crop production (e.g. citrus fruits, walnuts tree, etc.) (Eilers et al., 2011). In contrast, crops such as wheat and rice providing mainly macronutrients (e.g. carbohydrate) are generally wind or self-pollinated (Culley et al., 2002). Moreover, it has been estimated that honey bees are responsible for providing pollination service to 96% of animal-pollinated crops and thus playing a key role in the maintenance and reproduction of 52 out of 115 leading global commodities (Vanengelsdorp and Meixner, 2010; Klein et al., 2007). In Europe, 84% of 264 cultivated crops are pollinated by insects and 4.000 vegetable varieties depend on bee pollination services as well as in the production of fruits (e.g. kiwi, raspberries, blueberries, etc.), seeds and vegetables (e.g. sunflower seeds, beetroot, carrots) through pollination services (Williams, 1994; Corbet et al., 1991; Bommarco et al., 2012; Hoshide et al., 2018). Bees are also indirectly responsible for the reproduction and maintenance of wild plant communities and biodiversity (Aguilar et al., 2006; Ashman et al., 2004; De Groot et al., 2002). In addition, it is well known that managed honey bees provide honey, pollen, wax (e.g. for food processing), propolis (e.g. food technology), and royal jelly (used as a dietary supplement or as food ingredient) (Formato et al., 2011; Tinto et al., 2017). Overall, bees represent a very significant pollination service bridging agriculture, the food chain and the ecosystem thereby ensuring food production and security (Rose et al., 2015). In economic terms, the pollination services, from honey bees, bumble bees and wild bees contribute at least to 22 billion EUR each year of the European agriculture sector (Commission, 2016).

Over the last decade, important honey bee colony losses have been reported, particularly in North America and Western Europe (Jacques et al., 2016; Sanchez-Bayo and Goka, 2016; Steinhauer et al., 2014; Van der Zee et al., 2012). Scientific evidence shows that the weakening or death of bee colonies is mainly caused by the combined effects of multiple stressors rather than by one-off sudden attacks by a single factor (Goulson et al., 2015; EFSA, 2014a; Potts et al., 2010; Rortais et al., 2017). Such interactions can occur principally between (i) biological factors (Nazzi et al., 2012; Nazzi and Pennacchio, 2014), (ii) environmental factors (Di Pasquale et al., 2016; Goulson et al., 2015; Le Conte and Navajas, 2008), (iii) chemical and nutritional stressors (Tosi et al., 2017; Tong et al., 2019), (iv) chemical and biological factors (Williamson et al., 2013; Klein et al., 2017; Alaux et al., 2010; Vidau et al., 2011; Pettis et al., 2012; Renzi et al., 2016) and (v) multiple chemicals (EFSA, 2013a, b; Robinson et al., 2017; Han et al., 2019; Sanchez-Bayo and Goka, 2016). In particular, the latter is raising concerns among scientists and regulatory bodies since bees can be exposed to a wide range of multiple chemicals, “chemical mixtures”, including compounds from anthropogenic (e.g. plant protection products or veterinary drugs) or natural origin (e.g. mycotoxins, flavonoids, plant toxins) (Johnson, 2015; Tosi et al., 2019; EFSA PPR Panel, 2012; EFSA, 2014a). Hence, investigating the relative impact of multiple chemicals in comparison to non-chemical stressors (e.g. varroa, viruses) on bee health has been identified by the European Food Safety Authority (EFSA) as a priority to support the development of holistic risk assessment (RA) methods (EFSA AHAW Panel, 2016; EFSA, 2017a; Rortais et al., 2017). In this context, the Scientific Committee of EFSA has recently published a guidance document on “harmonised methodologies for human health, animal health and ecological RA of combined exposure to multiple chemicals” which provides a harmonised framework and step wise approaches for whole mixture and component-based approaches. The step wise approaches are applied to very step of the RA process namely problem formulation, exposure assessment, hazard identification and characterisation, risk characterisation and uncertainty analysis (More et al., 2019). When dealing with component-based approaches, two main mathematical reference models are usually applied when predicting combined toxicity assuming non-interaction: dose/concentration addition (CA) (Loewe, 1926) and independent action/response addition (IA) (Bliss, 1939). When combined toxicity significantly deviates from the observed responses from CA or IA, predictions are usually referred to and modelled as interactions (Jonker et al., 2005; Kienzler et al., 2016; More et al., 2019). Interactions have been described as either antagonism (i.e. combined toxicity is below the sum of the components’ toxicity) or synergism (i.e. toxicity of mixture greater than the sum of components’ toxicity) (Kienzler et al., 2014). However, if only one of the chemicals in the binary mixture is expected to cause adverse effect (e.g. clothianidin + piperonyl butoxide), synergism is usually defined as potentiation (Heys et al., 2016; Robinson et al., 2017). In practice, mixtures of components with similar Modes of Action (MoA) are addressed using the CA model, whereas compounds with different MoAs are assessed using the IA model that mathematically combine probabilities of independent events (Jonker et al., 2005; Belden et al., 2007). Overall, evidence from the literature and scientific advisory bodies worldwide support the application of CA as a conservative approach compared to IA unless evidence for interactions can be demonstrated (Bopp et al., 2015; EFSA, 2013a, b; More et al., 2019).

The current manuscript provides the first quantitative review of the available laboratory toxicological studies of binary mixtures of chemicals (i.e. pesticides, veterinary drugs and environmental contaminants) in honey bees and wild bees. It aims to support hazard assessment by means of extensive literature searches, data collection, modelling and analysis of combined toxicity (dose addition, interactions (i.e. synergism, antagonism)) and their associated mechanisms. First, extensive literature searches are perfomed to identify and collect combined toxicity endpoints (e.g. LD_{50} or LC_{50}) from acute and chronic laboratory studies on binary mixtures in honey bees and wild bees (solitary bees and bumble bees) together with available toxicity data and mode of action information from public databases. In addition, dose response from each individual binary mixture experiment are modelled to identify the nature and potency of the combined toxicity (dose addition, synergism, antagonism) and quantify its magnitude using a toxic unit approach and the MIXTOX model. Furthermore, new predictive hazard assessment tools applicable to large binary mixture datasets in bees are developed. The reader should note that exposure assessment (pesticides application rate, crop management, consumption patterns, etc.) and full risk characterisation are beyond the scope of this quantitative analysis. Implications for risk assessment and future directions concludes while considering mechanisms of interactions, data gaps, importance of exposure assessment scenarios and risk characterisation as well as the development of methods to assess multiple chemicals and multiple stressors in bees to support risk management.

2. Materials and methods

2.1. Extensive literature searches

Extensive Literature Searches (ELS) were performed by two independent reviewers in January 2018 to critically appraise, collect and analyse data on toxicity of mixtures in bee species (EFSA, 2010), using structured search strategies (Appendix S1). ELSs were carried out in PubMed (1975–2018), in Web of Science Core Collection (1975–2018), including Science Citation Index Expanded, CABI: CAB Abstracts®, Current Contents Connect®, Data Citation Index SM, FSTA® the food science resource, MEDLINE®, SciELO Citation Index, Zoological Record®, Conference Proceedings Citation Index- Science, Book Citation

Index– Science, Current Chemical Reactions, Index Chemicus). All records were computed in the EndNote™ software. In addition, bibliographical sources from EFSA studies and database on mixture toxicity in bees were checked thoroughly for completeness (Quignot et al., 2015; Robinson et al., 2017). In addition, qualitative information on the Mode of Action (MoA) of the individual chemicals were collected from the literature and available databases (Sparks and Nauen, 2015; Hermann and Stenzel, 2019; Sanchez-bayo, 2012; Johnson et al., 2012, 2013; Leroux et al., 2008; De Castro et al., 2015; Huang et al., 2013).

Each individual publication retrieved in EndNote™ libraries was screened and assessed using inclusion and exclusion criteria reported in Table 1 in two steps (i) screening of the titles and abstracts and (ii) screening of the full-text of the publications. All included and excluded publications are available under individual EndNote™ libraries.

2.2. Data collection and analysis

2.2.1. Data collection

Following the Extensive Literature Searches, individual toxicological endpoints (acute and chronic) from laboratory mixture experiments (e.g. LD_{50} or LC_{50}) were collected for the oral and contact exposure according to the inclusion criteria, including bee species, sample, size, summary statistics (mean, median, standard error of the mean, standard deviation, confidence intervals) and exposure patterns. Standardised templates were developed to structure the data into an excel database designed with relevant picklists. When papers reported only graphical information, quantitative data were extracted using "Plot Digitizer GNU" software (available at: http://plotdigitizer.sourceforge.net/) or the R software (R Core Team, 2019).

In addition, reference points (e.g. LD_{50}, LC_{50}) for all individual chemicals i.e. mostly Plant Protection Products (PPPs) in honey bees were extracted from EFSA's Chemical Hazards database "OpenFoodTox" (available at: https://zenodo.org/record/1252752#.XLg-4Oj7SUm) (Dorne et al., 2017; EFSA, 2014b) and other publicly available databases were consulted including the US-EPA dashboard (https://cfpub.epa.gov/ecotox/), OECD e-ChemPortal (https://www.echemportal.org/echemportal/index.action), PPDB-Pesticide Properties Database (https://sitem.herts.ac.uk/aeru/ppdb/). All binary mixtures data were compiled in an excel database for further analysis (see Section 2.2.2).

2.2.2. Quantification of magnitudes of interaction

2.2.2.1. Estimated mean ratios. A comprehensive analysis of magnitude of interactions (as potency or synergism ratios) was performed through the calculation of Estimated Mean Ratios (EMR) for each individual single compound and binary mixture toxicity dataset or for combined toxicity between a single chemical and a non-chemical stressor (biological or nutritional). EMR has been defined as the ratio between the estimated mean toxicity (e.g. LD_{50}, LC_{50}, EC_{50}) of a given single chemical (chemical A) for which the experimental dose is available (EM_A) and the estimated toxicity of the binary mixture chemical A + chemical B (EM_M) or chemical A + non-chemical stressor (Quignot et al., 2015):

$$EMR = \frac{EM_A}{EM_M} \quad (1)$$

Each EMR for a given binary mixture (EM_M) is expressed on a harmonised scale starting at 1 to reflect changes in combined toxicity either as an increase (+) or a decrease (–) (Quignot et al., 2015).

It is noted that the EMR approach assumes that chemical B does not contribute to the mixture toxicity which does not fully comply with the principles of concentration addition (CA), which assumes that any amount of a chemical always contributes to the combined toxicity expressed in Toxic Units (Jonker et al., 2005). For each binary mixture, the statistical significance of the combined toxicity has been estimated using non-overlapping 95% confidence intervals (95% CI of the EM_A vs 95% CI of the EM_M for chemical A + B) as described in Johnson et al. (2012, 2013). All calculations were carried out in the R software (R Core Team, 2019).

In addition, risk of bias was assessed through the quantification of the variability across studies by calculating the Confidence Intervals (CIs) for each Estimated Mean Ratio (EMR). 95% CI were calculated according to the Fieller (1954) and Delta methods as described in the formulas (2), (3) and (4).

Fieller's method (Fieller, 1954) is based on the assumption that $(\hat{\theta}_1, \hat{\theta}_2)$ follows a bivariate Normal distribution. For testing $\theta_1/\theta_2 = R_0$ (which amounts to testing $\theta_1 = R_0\theta_2$), the two-sided *t*-test is based on:

$$(\hat{\theta}_1 - R_0\hat{\theta}_2)/\mathrm{Var}[\hat{\theta}_1 - R_0\hat{\theta}_2]^{1/2} \quad (2)$$

The rejection region for this test is the set of values r satisfying:

$$(\hat{\theta}_1 - r\hat{\theta}_2) > t\mathrm{Var}[\hat{\theta}_1 - r\hat{\theta}_2]^{1/2} \quad (3)$$

Finding an explicit form for the confidence interval requires solving a quadratic equation in r. The confidence interval can be of the form(L, U), $(U, +\infty)$ $(0, U)$ or $(0, +\infty)$ depending on the number of solutions of the quadratic equation (Raftery and Schweder, 1993; Buonaccorsi and Iyer, 1984; Franz, 2007; Von Luxburg and Franz, 2009; Hirschberg and Lye, 2010).

The Delta method is based on a Taylor series approximation of:

$$\hat{R} = \hat{\theta}_1/\hat{\theta}_2 \quad (4)$$

Around θ_1/θ_2 that is used to obtain estimates of the expectation and of the variance of $\hat{R}$ (Casella and Berger, 2002; Faraggi et al., 2003; Franz, 2007; Hirschberg and Lye, 2010). Assuming that $\hat{R}$ follows a

Table 1
Inclusion and exclusion criteria for the selection of relevant literature in the extensive literature search.

Inclusion criteria	
Review question	- Does the study provide toxicological outcomes for binary mixtures in bee species?
Population of interest	- Bees species (i.e. honey bees, bumble bees, *Osmia* spp.)
Study design	- *In vivo* experimental laboratory studies - *In vivo* field/semi-field studies; - Routes of exposure (i.e. contact, oral) - Study length (acute, chronic)
Outcome of interest	- Summary statistics or individual datasets on toxicity of mixtures and non-chemical stressors (e.g. LD_{50}, LC_{50} and related statistical descriptors) for single doses. - Summary statistics or individual datasets for multiple doses (dose response data) on toxicity of mixtures (e.g. LD_{50}, LC_{50} and related statistical descriptors)
Exclusion criteria	
Type of study	- *In vitro* studies - Studies reporting only qualitative data with no toxicological outcome; - Duplicated studies: studies reporting the same dataset in several publications, studies on non-chemical stressors or combined chemical and non-chemcial stressors, studies reporting results from systematic reviews, meta-analyses or predictive models.

normal distribution, the $1-\alpha$ confidence interval is obtained as $\hat{R} \pm z \times$ s. d. where z is the upper $\alpha/2$ quantile of the standard normal distribution.

2.2.2.2. Standardised mortality ratios. For combined toxicity data reporting mean mortality or survival probability expressed in % of individual bees, the standardised mortality ratios (SMR) has been estimated as the ratio or percentage change in observed deaths compared to that occurring after exposure to the single compound. An SMR above 1 is simply interpreted as a higher number of observed deaths compared to the group exposed to the single compound (Everitt and Skrondal, 2010).

2.2.2.3. Toxic Unit approach. Analysis of each experimental binary mixture for acute and chronic (contact or oral) toxicity was conducted using the Toxic Unit approach to standardise applied dose and critical endpoints (i.e. LD_{50}) using matching datasets for each chemical from OpenFoodTox and other databases. The toxic unit approach assumes that mixture toxicity predictions follow the Dose/Concentration Addition (DA/CA) model given the quantitative composition of each chemical within the mixture in relation to their relative potency (Jonker et al., 2005). Toxic Unit for chemical B (i.e. TU_B) is given as the ratio of the dose/concentration of chemical B applied in the binary mixture experiment relative to the selected critical endpoint (e.g. LD_{50}) used as reference as follows:

$$TU_B = \frac{\text{Applied Dose}_B}{\text{Critical Endpoint}_B} \quad (5)$$

$TU_B = 0.1$ indicates that the dose of compound B applied in the mixture assay corresponds to 10% of the LD_{50} or LC_{50}. The expected combined potency of the mixture relative to a given acute (e.g. LC_{50}, LD_{50}) or chronic (e.g. long-term NOEC) toxicological endpoint is also named "mixture strenght" or mixture potency symbolised as "TUm" (More et al., 2019) according to Eq. (6) (Jonker et al., 2005):

$$TUm = \sum_{i=1}^{n} \frac{C_i}{ECx_i} \quad (6)$$

Effect Concentrations (ECx_i) relate to the critical endpoint selected as reference, and Concentration (Ci) refers to the concentration of the chemical (i) in the mixture. Consequently, while assuming CA as the default reference model, TUm is calculated by summing the individual TU_i values for each compound present in the mixture (binary, ternary or with more components) (SCCS, SCENHIR and SCHER, 2012; More et al., 2019) as follows:

$$TUm = \sum_{i=1}^{n} TU_i \quad (7)$$

A mixture with a TUm = 1 would be expected to produce the effect used as the critical endpoint in the TU calculations (e.g. EC_{10} = > 10% effect, EC_{50} = > 50% effect, LC_{50} = > 50% lethality).

In addition, individual TU_B values were ranked into three classes in comparison with their corresponding EMR to plot and quantify the relative contribution of compound B (TU_B) to the overall combined mixture (TUm) (see results, 3.2.2):

- $TU_B \leq 0.10$
- $0.11 \leq TU_B \leq 0.30$
- $0.31 \leq TU_B \leq 0.60$

According to each TU_B class, the distribution of the EMR values against their "reverse cumulative frequency" has been plotted and fits were tested with Pearson product-moment correlation coefficient (R^2). This allowed quantifying the contribution of chemical B to the combined toxicity of the binary mixture.

2.2.3. Predictive models for combined toxicity and model deviation ratios

For each individual binary mixture, predictive models of combined toxicity were compared to the experimental dose response data to assess deviation from DA/CA, i.e. interactions synergism, potentiation or antagonism. The DA/CA model assumes that the chemicals have a similar Mode of Action (MoA) in the mixture and they do not interact with each other, thus that they do not influence each other's uptake, distribution or metabolism at the site of the biological target (Faust et al., 2003; Jonker et al., 2005; Cedergreen et al., 2014, 2012; Backhaus et al., 2004, 2013).

Therefore, if a mixture of n chemicals with TUm = 1 results in an x % (i.e. the selected critical endpoint reference value) effect compared to the control response, then the mixture is acting according to DA/CA as the following relationship holds:

$$TUm = \Sigma_{i=1}^{n} \frac{C_i}{ECx_i} = 1 \quad (8)$$

where Ci represent the concentration of chemical i in the mixture and ECx_i is the effect concentration of chemical i that results in the same effect (x%) as observed in the mixture. However, as full dose response data are rarely reported in the literature it is difficult to derive all EC_x values to test mixtures yielding effects of different intensity (e.g. 10, 20, 50, and 80%). Hence, because the most commonly reported critical endpoint values usually refer to 50% effects for both single chemicals and mixtures, the expected TUm for a mixture observed to give 50% mortality under CA would be TUm = 1, when the TU_i values are using the LC_{50} values of the individual chemicals as reference values. Based on the availability of critical endpoints from the data collection, EC_x in Eqs. (6) and (8) were substituted with LC_{50} or LD_{50} values to quantify how well the observed effects fit the CA predictions for the binary mixture toxicity in bee species.

The magnitude of the deviation between the concentration addition-predicted model (predicted TUm) and the experimental data (observed TUm) was calculated as model deviation ratio (MDR) based on the TUm values according to Belden et al. (2007) using Observed TU values calculated as TUm in the mixture (50% mortality) compared to that from the expected TUm value of a mixture causing 50% lethality as TU of 1 as follows:

$$MDR = \frac{\text{predicted } TU_m}{\text{observed } TU_m} \quad (9)$$

Here, MDR values (Eq. (9)) represent the ratio between the expected or "predicted TUm" for a binary mixture causing 50% mortality (by definition a TUm = 1) (Eq. (8)), and the "observed TUm" (Eq. (6)) calculated as TUm causing 50% mortality (Belden et al., 2007; Coors and Frische, 2011; Cedergreen et al., 2013, 2012). Thus, MDR values above 1 indicates toxicity above that expected from CA predictions, and MDR values below 1 indicates toxicity below that expected from CA predictions. According to the current scientific literature (Belden et al., 2007; Cedergreen, 2014), biologically significant synergism has been defined for a range of species as a deviation from CA superior to two-fold. As a consequence, mixtures are usually termed additive for $0.5 \leq MDR \leq 2$, antagonistic for MDR values < 0.5 and synergistc for MDR values > 2 (Belden et al., 2007; Cedergreen, 2014). In our analysis, besides applying the MDR approach to characterise mixture effects, the statistical significance of the combined toxicity was assessed and calculated using non-overlapping 95% confidence intervals (i.e. 95% CI of the EM_A vs 95% CI of the EM_M for chemical A + B) as described by Johnson et al. (2012, 2013). From this analysis of statistical significance, MDR thresholds were refined as follows:

- MDR values between 0.83 and 1.25 indicate that combined toxicity follows **DA/CA** with observed TUm values deviating less than 1.5-fold from the expected TUm of 1.
- MDR values < 0.83 indicate that combined toxicity is below that predicted from CA and classified as **antagonism;**

- MDR > 1.25 indicates that combined toxicity is above that predicted from CA and classified as **synergism.**

2.2.4. Comparison of estimated mean ratios and model deviation ratios

A polynomial regression model (with formula y ~ x + I(x^2) + I (x^3)) was fitted between the EMR from the individual dose response data and the corresponding individual MDR to assess the potential correlation between the two approaches by means of a Pearson product-moment correlation coefficient (R^2) (see result 3.2.4). R software has been used and R-script is provided in supplementary materials.

3. Results and discussion

3.1. Extensive literature searches

The results of the extensive literature search on combined toxicity of chemicals in honey bees and wild bees (solitary bees and bumble bees) are illustrated in Fig. 1 as a PRISMA flow diagram. 957 peer-reviewed articles were initially identified from the literature with total of 14 papers matching the inclusion criteria with relevant data providing a total of 218 binary mixtures (Moher et al., 2009) resulting from *in vivo* experimental laboratory studies. Overall, most publications (n = 10) reported mortality data in honey bees (*Apis mellifera*) for binary mixtures of pesticides with dose response data available for a total of 92 individual binary mixtures (Johnson et al., 2013, 2012, 2009, 2006; Zhu et al., 2017; Guseman et al., 2016; Rinkevich et al., 2015; Wilkinks et al., 2013; Iwasa et al., 2004; Ellis et al., 1997). Similarly, four peer-reviewed articles provided relevant data for binary mixture toxicity in wild bees (*Bombus* spp.) and solitary bees (*Osmia* spp.) (Robinson et al., 2017; Sgolastra et al., 2017; Spurgeon et al., 2016; Biddinger et al., 2013). Finally, studies on chemical-non-chemical interactions were provided in two peer-reviewed articles and were excluded from the analysis (Tosi et al., 2017; Alaux et al., 2010).

Overall, toxicity data were mostly available for acute contact toxicity i.e. topical application (61%) with few studies reporting chronic oral effects (20%) or acute oral toxicity data (19%) as highlighted in a recent meta-analysis (Quignot et al., 2015). The rationale behind such findings lies in the fact that acute toxicity tests (24 and 48 h) for honey bees are usually applied in the area of chemical risk assessment for regulated products such as pesticides. However, honey bees are exposed chronically to a range of chemicals (both alone and in combination), either by foraging on contaminated areas, or through contaminated food, stored and consumed in the hive (EFSA, 2013a; EFSA AHAW Panel, 2016). Recently, the OECD (Organisation for Economic Co-operation and Development) proposed a new guideline (OECD, 2017) for chronic oral toxicity tests (10-days feeding test in the laboratory).

From the extensive literature search, data for 51 chemicals, the vast majority as pesticides, were identified and their corresponding Modes of Action (MoA) were analysed for their pesticidal MoA for 23 insecticides and 16 fungicides respectively as well as MoA in honey bees as non-target species (Table 2). For the toxicological MoA in honey bees, classifications schemes from the Insecticide Resistance Action Committee (IRAC) and the Fungicide Resistance Action Committee's (FRAC) covering the specific target sites in target organisms for insecticides, acaricides and fungicides and the scientific literature were reviewed (Leroux et al., 2008; Hermann and Stenzel, 2019; Sanchez-bayo, 2012; Johnson et al., 2012, 2013; Huang et al., 2013; de Castro et al., 2015; Sparks and Nauen, 2015). In this context, pyrethroids/

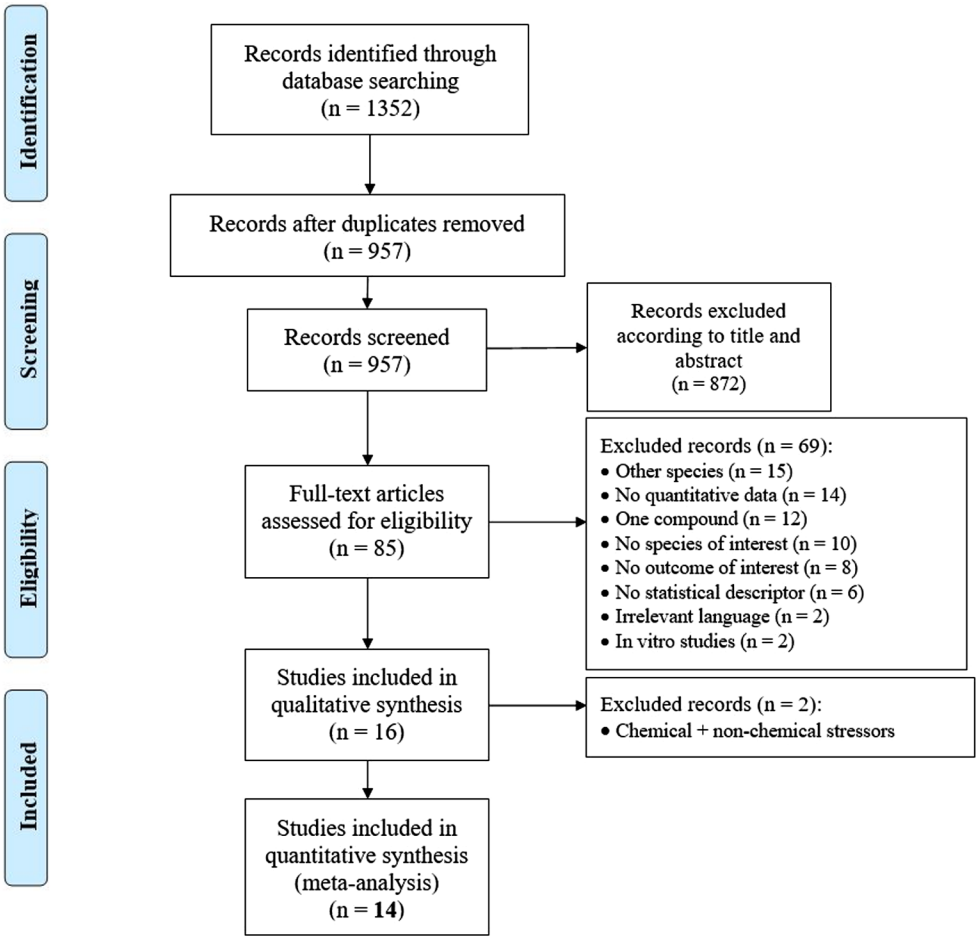

Fig. 1. PRISMA 2009 Flow Diagram for the extensive literature searches on combined toxicity of binary mixtures in bee species.

Table 2
Overview of xenobiotics with available binary mixture toxicity data in bees: class, chemical group and Mode of action (MoA) (Leroux et al., 2008; Hermann and Stenzel, 2019; Sanchez-bayo, 2012; Huang et al., 2013; Sparks and Nauen, 2015; Johnson et al., 2012, 2013; de Castro et al., 2015).

Compounds	Class of compounds	Chemical group		MoA
		Name	Code[1]	
Amitraz	Insecticide	Amitraz/formamidine	19	Octopamine receptor agonists (Nerve action)
Carbaryl	Insecticide	Carbamates	1A	Acetylcholinesterase (AChE) inhibitors (Nerve action)
Oxamyl				
Acephate	Insecticide	Organo-phosphates	1B	Acetylcholinesterase (AChE) inhibitors (Nerve action)
Coumaphos				
Dimethoate				
Fenpyroximate	Insecticide	METI acaricides and insecticides	21A	Mitochondrial complex I electron transport inhibitors (Energy metabolism)
Aldrin	Insecticide	Organo-chlorine	2A	GABA-gated chloride channel antagonists
Dieldrin				
Bifenthrin	Insecticide	Pyrethroids/Pyrethrins	3A	Sodium channel modulators (Nerve action)
Cyfluthrin				
Fluvalinate				
Lambda-cyhalothrin				
Tau-fluvalinate				
Phenothrin				
Acetamiprid	Insecticide	Neonicotinoid	4A	Nicotinic acetylcholine receptor (nAChR) agonists (Nerve action)
Clothianidin				
Imidacloprid				
Thiacloprid				
Thiamethoxam				
Sulfoxaflor	Insecticide	Sulfoximines	4C	Nicotinic acetylcholine receptor (nAChR) agonists (Nerve action)
Oxalic acid	Insecticide	Natural insecticide	NA	NA
Azoxystrobin	Fungicide	Methoxy-acrylates (Strobilurin)	C3	Complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site (cyt b gene) (Respiration)
Boscalid	Fungicide	Pyridine-carboxamides	NA	Complex II: succinate-dehydrogenase (Respiration)
Epoxiconazole	Fungicide	Triazoles	NA	C14- demethylase in sterol biosynthesis (erg11/cyp51) DMI-fungicides (DeMethylation Inhibitors)
Fenbuconazole (Indar)				
Metconazole				
Myclobutanil				
Propiconazole				
Tebuconazole				
Tetraconazole				
Triadimefon				
Uniconazole-P				
Triflumizole	Fungicide	Imidazoles	NA	C14-demethylase in sterol biosynthesis (erg11/cyp51) DMI-fungicides (DeMethylation Inhibitors)
Prochloraz				
Chlorothalonil	Fungicide	Chloronitriles	NA	Multi-site contact activity
Pyraclostrobin	Fungicide	methoxy-carbamates	C3	complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site (cyt b gene) QoI-fungicides (Quinone outside Inhibitors)
Glyphosate	Herbicide	Organophosphorus	NA	Enzyme inhibitor (it disrupts the shikimic acid pathway through inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase)
Diethyl maleate (DEM)	Chemical/ Synergist	NA	NA	Enzyme inhibitor Glutathione S-transferase (GST) inhibitor
Piperonyl butoxide (PBO)	Chemical/ Synergist	Cyclic aromatic	27A	Enzyme inhibitor Blocks pests natural detoxification system (P450-dependent monooxygenase inhibitor)
S,S,S-tributyl phosphorotrithioate (DEF)	Chemical/ Synergist	Organo-phosphorus	NA	Carboxylesterase inhibitor
Fumagillin	Veterinary products/drug	Antimicrobial agent	NA	Enzyme inhibitor (methionine aminopeptidase2 - MetAP2)
Ivermectin	Veterinary product/drug	Avermectins	NA	Receptor disrupter (γ-aminobutyric acid receptors, GABA-R)
Oxytetracycline	Veterinary products/drug	Antibiotic	NA	NA
Phenobarbital	Chemical	Barbituric acid derivate	NA	Receptor disrupter (γ-aminobutyric acid receptors, GABA-R)
Quercetin	Flavonoid	Flavonoid (polyphenol)	NA	Mammalian P-glycoprotein inhibitor
Salicylic acid	Acaricide (organic)	NA	NA	Cox inhibition Anti-inflammatory
Thymol	Veterinary product/drug	Monoterpenoid phenol	NA	Ergosterol biosynthesis disrupter
Tylosin	Veterinary product/drug	Antimicrobial agent	NA	Bacteriostatic
Verapamil	Drug	NA	NA	P-glycoprotein transport modulator
Xanthotoxin	Chemical	Furanocoumarin (produce by plants)		Enzyme inhibitor (xenobiotic-metabolizing P450s)

[1] = Code of chemical group name according to IRAC/FRAC classification shemes.

pyrethrins insecticides and conazole fungicides were the most investigated pesticides (≈55%) belonging to the MoA groups of "sodium channel modulators" (≈25%) and "demethylation inhibitors" (≈30%) respectively, and amongst conazoles, triazole fungicides (Demethylation Inhibitors) provided the largest experimental datasets for binary mixtures in honey bees. Similarly, the combined exposure to neonicotinoid insecticides (Nicotinic acetylcholine receptor agonists) and conazole fungicides were the second most investigated mixtures (35%).

3.2. Data collection and analysis

3.2.1. Data collection

218 individual binary mixtures were collected and included in the statistical analyses with the majority of toxicological endpoints reported as lethal doses or concentrations (e.g. LD_{50}, $LC_{50,}$) for pesticides or pesticides and veterinary drugs combinations with 133, 44 and 41 mixtures reporting acute contact toxicity (i.e. topical application), chronic oral toxicity and acute oral toxicity, respectively (tables S1, S2, S3, S7). Combined toxicity data for binary mixtures were available as dose response data in honey bees species (Johnson et al., 2009, 2012, 2013) for acute contact toxicity (n = 92) and acute oral toxicity (n = 15) (tables S3, S7). All toxicity data are available as spreadsheets on EFSA knowledge junction under the DOI: https://doi.org/10.5281/zenodo.3383713 and as summary tables in supplementary materials (Tables S1 – S11) classified according to route and exposure patterns (i.e. oral, contact, acute and chronic) and toxicological endpoints (e.g. LD_{50}, LC_{50}) for the honey bees (*Apis mellifera*) and wild bee species *(Osmia bicornis, Bombus terrestris*). All the studies included in this meta-analysis were performed *in vivo* experimental laboratory tests according to standard toxicity tests as provided by the author(s). In honey bees, acute contact toxicity studies refer to "topical application" and toxicity tests were mainly conducted on group feeding tests (Johnson et al., 2013, 2009, 2006; Iwasa et al., 2004; Biddinger et al., 2013). Similarly, acute oral studies on honey bees and bumble bees were conducted on group feeding tests through consumption of contaminated food (e.g. nectar, pollen) (Robinson et al., 2017; Johnson et al., 2012). In contrast, toxicity studies on solitary bees such as *Osmia* spp. were conducted on individual feeding tests (Sgolastra et al., 2017; Biddinger et al., 2013).

3.2.2. Quantification of magnitudes of interaction

3.2.2.1. Estimated mean ratios. EMRs were calculated to characterise the magnitude of the combined toxicity for each individual binary mixture and expressed on a harmonised scale starting at 1 to reflect changes in the toxicological endpoint (EM_M) either as an increase (+) or a decrease (–) in combined toxicity (Quignot et al., 2015).

3.2.2.1.1. Acute contact toxicity. The acute contact toxicity database represented the largest database in honey bees with 133 LD_{50} for binary mixtures including dose response data (n = 92) (Tables S1–S3 and S15). A comprehensive analysis of the database provided an analysis of Toxic units below, prediction of combined toxicity and calculation of MDRs in Section 3.2.2 and comparison of EMRs and MDRs in Section 3.2.3. Overall, EMRs for binary mixtures reflecting statistically significant interactions (non-overlapping 95% CI) were highest (> 100) for honey bees exposed to neonicotinoid insecticides (e.g. acetamiprid, thiacloprid) combined with cytochrome P450 (CYP) inhibitors (e.g. triazole fungicides such as propiconazole) and synergists (e.g. piperonyl butoxide (PBO) (Table S3). Examples include EMRs of 1980 for pyrethroid tau-fluvalinate and prochloraz (TU_B = 0.07) and PBO (TU_B = 0.03) as well as EMRs of 235- and 101-fold for the neonicotinoid acetamiprid-triflumizole (TU_B = 0.50) and acetamiprid-propiconazole (TU_B = 0.10) (Table S3). In contrast, reduced combined toxicity through antagonistic interactions were also observed in a few instances (e.g. amitraz-oxalic acid, 4-fold) (Table S3).

3.2.2.1.2. Acute oral toxicity. EMR values were calculated for the 41 LC_{50} binary mixtures available for pesticides and veterinary drugs (Tables S4–S8). For honey bees, EMR values reflecting increase in combined toxicity were statistically significant for tau-fluvalinate (pyrethroid) with xanthotoxin (furanocoumarin produced by plants) with a value of ≈200 (Johnson et al., 2012), phenobarbital with

Table 3
Ranking of combined toxicity for binary mixtures of pesticides and veterinary drugs in honey bees (expressed as LD50 μg/bee) following acute oral exposure (Johnson et al., 2013; 2012; Wilkins et al., 2013). Estimated Mean Ratio (EMR) for the binary mixture (chemical A + B) relative to chemical A alone as well as Confidence Interval (CI 95%) for EMR are provided.

Study_ID	Chemical A		Binary Mixture (A + B)								
	Name	EM_A	Chemical B	TU_B	EM_M	CI_1 (95th)	CI_2 (95th)	EMR (+)	EMR (–)	CI_{EMR}	Slope (± SE)
Study_165	Tau-Fluvalinate[2]	8.1 (7.2–9.0)	Xanthotoxin	NA	0.04	0.001	0.13	201		na	0.3 ± 0.09
Study_164	Tau-Fluvalinate[2]	8.1 (7.2–9.0)	Phenobarbital	NA	0.19	0.12	0.31	42*		20.7–64.0	1.5 ± 0.12
Study_169	lambda-cyhalothrin[2]	0.048 (0.034–0.068)	Phenobarbital	NA	0.02	0.005	0.025	2.8*		0.67–4.1	2.9 ± 0.4
Study_151	Tau-fluvalinate[1]	9.2 (7.9–10.8)	Fumagillin	NA	4.8	3.7	6.32	1.9[a]		1.3–2.5	2.0 ± 0.22
Study_167	Tau-Fluvalinate[2]	8.1 (7.2–9.0)	Salicylic acid	NA	4.5	2.2	8.6	1.8		0.49–3.1	1.6 ± 0.33
Study_171	Dieldrin[2]	0.037 (0.032–0.047)	Phenobarbital	NA	0.02	0.01	0.03	1.8*		0.86–2.9	3.5 ± 0.30
Study_170	Aldrin[2]	0.061 (0.0527–0.071)	Phenobarbital	NA	0.04	0.03	0.05	1.6*		1.1–2.0	3.9 ± 0.36
Study_163	Thymol[1]	38.1 (27.3–49.6)	Fumagillin	NA	25.3	21.3	29.7	1.5		0.99–2.0	4.0 ± 0.41
Study_158	Amitraz[1]	5.47 (4.12–7.1)	Oxytetracycline	NA	3.7	3.0	4.7	1.5		0.96–2.0	4.2 ± 0.54
Study_160	Amitraz[1]	5.5 (4.1–7.1)	Fumagillin	NA	3.9	2.9	5.2	1.4		0.85–2.0	3.8 ± 0.52
Study_161	Thymol[1]	38.1 (27.3–49.6)	Oxytetracycline	NA	27.5	15.4	45.1	1.4		0.53–2.2	3.6 ± 0.85
Study_152	Coumaphos[1]	26 (19.5–39.5)	Oxytetracycline	NA	20	15.1	27.6	1.3		0.65–1.9	2.5 ± 0.36
Study_159	Amitraz[1]	5.5 (4.1–7.1)	Tylosin	NA	4.5	3.8	5.3	1.2		0.82–1.6	3.3 ± 0.29
Study_162	Thymol[1]	38.1 (27.3–49.6)	Tylosin	NA	32.3	14.5	47.7	1.2		0.48–1.9	3.1 ± 0.75
Study_153	Coumaphos[1]	27 (19.5–39.5)	Tylosin	NA	25.7	17.9	43.0	1.		0.38–1.6	3.5 ± 0.72
Study_149	Tau-fluvalinate[1]	9.2 (7.95–10.8)	Oxytetracycline	NA	8.4	7.3	9.8	1.[b]		0.85–1.3	2.7 ± 0.21
Study_155	Fenpyroximate[1]	3.2 (2.7–3.9)	Oxytetracycline	NA	4.7	3.9	5.7	0.69[a]	1.5	1.1–1.8	3.5 ± 0.37
Study_157	Fenpyroximate[1]	3.24 (2.7–3.9)	Fumagillin	NA	5.5	4.4	6.9	0.59[a]	1.7	1.2–2.2	2.8 ± 0.32
Study_168	Tau-Fluvalinate[2]	8.1 (7.2–9.0)	Indole-3-carbinol	NA	8.3	5.9	10.9	0.97	1.03	0.70–1.4	2.5 ± 0.67
Study_150	Tau-fluvalinate[1]	9.2 (7.9–10.8)	Tylosin	NA	10.5	8.1	14.9	0.88	1.1	0.73–1.6	2.3 ± 0.34
Study_156	Fenpyroximate[1]	3.24 (2.7–3.9)	Tylosin	NA	4.1	3.6	4.6	0.80	1.3	0.97–1.5	2.6 ± 0.18
Study_154	Coumaphos[1]	28.0 (19.5–39.5)	Fumagillin	NA	33.3	25.5	49.2	0.78	1.3	0.60–1.9	2.1 ± 0.28
Study_166	Tau-Fluvalinate[2]	8.1 (7.2–9.0)	Quercetin	NA	11.4	9.7	13.9	0.71	1.4	1.1–1.7	3.0 ± 0.40

1 = Johnson et al., 2013 (tau-fluvalinate + sucrose): significant differences compared to the respective treatment are indicated with a superscript letter "a" = significant pre-treatment effect, "b" = significant pre-treatment*acaricide dose effect. 2 = Johnson et al., 2012: treatments with non-overlapping 95% confidence interval are considered significantly different. CI = 95% Confidence Interval.

lambda-cyhalothrin, aldrin and dieldrin with a 2.8-, 1.6- and 1.8-fold increase in combined toxicity respectively (Table 3). For veterinary products, EMRs also showed an increase in combined toxicity for ivermectin ($p < 0.0001$) with verapamil (EMR = 4.1) > quercetin (EMR = 2.6) > fumagillin (EMR = 1.8) (Guseman et al., 2016) (Tables S5 and S6). In contrast, a slight decrease (1.5–1.7 fold) in combined toxicity of fenpyroximate (METI-acaricide) with oxytetracycline and fumagillin (veterinary products) was observed (Table S8).

Sgolastra et al. (2017) investigated combined toxicity, expressed as standardised mortality ratios (SMR), after exposure to binary mixtures of pesticides in three bee species (*A. mellifera, B. terrestris, O. bicornis*) at different time points (Table S7) and found significant synergistic mortality in all species exposed to non-lethal doses of propiconazole ($TU_B = 0.07$) and respective LD_{10} of the neonicotinoid insecticide clothianidin ($TU_A = 0.10$). Such a significant increase in combined toxicity was measured for acute time points in *A. mellifera* (4 h and 24 h) and *B. terrestris* (4 h), these persisted throughout the experiment (96 h) in *O. bicornis*. Overall, SMR the magnitudes of synergism ranged from 4.4-fold in *A. mellifera* (at 24 h) to 8.7 in *O. bicornis* (at 4 h) (Table S7).

3.2.2.1.3. Sub-chronic and chronic oral toxicity. EMR values for subchronic (LC_{50} 96 h) and chronic (LC_{50} 240 h) mortality (n = 44) after exposure to pesticide binary mixtures are provided in supplementary materials (Tables S9–S13). Overall, EMRs for subchronic toxicity increased by a maximum of 1.5-fold in *A. meliferra* and *B. terrestris* whereas an EMR of 8.6-fold was reported for *O. bicornis* (Sgolastra et al., 2017) (Table S7).

Chronic oral toxicity (LC_{50} 96 h, 240 h) of binary mixtures of pesticides (in *Apis mellifera*, *Bombus terrestris* and *Osmia bicornis*) showed an increase in toxicity with exposure time for all tested chemicals (n = 6) (Table S9; Fig. 2). In particular, effects on mortality increased from the 48 h time interval until 240 h exposure time by 1.3–1.6-fold in *B. terrestris* and *O. bicornis,* respectively (Robinson et al., 2017). Combined toxicity of tau-fluvalinate (pyrethroid) and propiconazole (SBI fungicide) showed potentiation via inhibition of metabolism by SBI fungicides (Berenbaum and Johnson, 2015; Han et al., 2019). However, potentiation effects between SBI fungicides and clothianidin were not observed, and recent findings demonstrated that the expression of clothianidin induces the *CYP9q1* detoxification gene by (Yao et al., 2018). Zhu et al. (2017) show that none or very minor additive toxicity was for 5 binary mixture of imidacloprid and other pesticides (i.e. lambda-cyhalothrin, oxamyl, tetraconazole, glyphosate, sulfoxaflor) at concentrations similar to the residue levels detected in honey bee hives (i.e. field concentration) (Table S10). However, the author did not exclude that synergism may occur under other exposure situations particularly at higher concentrations or with different proportions of individual chemicals.

Combined chronic sub-lethal effects of coumaphos (organo-phosphate acaricide) and prochloraz (imidazole fungicide) in honey bee workers were investigated with regards to the molecular immune response at different developmental stages (prepupa, white-eyed pupa, adult) (Cizelj et al., 2016). Changes in mRNA level associated with upregulation of a range of genes (e.g. abaecin, defensin-1, cactus and basket) were reported for prochloraz and coumaphos. In addition, our results on mortality data suggest that an increased toxicity (EMR = 70) is observed when adult bees are exposed to coumaphos-prochloraz mixture, thus highlighting strong synergistic effects (MDR = 12.5) (Table S3; Fig. 2).

3.2.2.1.4. Acute and chronic oral toxicity of multiple stressors

Toxic Unit approach

As described in the method Section 2.2.2, the Toxic Unit (TU) approach has been applied to quantify potency of the binary mixture A + B (TUm) versus compound A (TU_A). In addition, the dose of compound B in each experiment (TU_B) has been estimated using matching potency information ($LC_{50\text{-}B}$) binary mixtures from available databases (EFSA PPR, 2012; SCCS, SCENHIR and SCHER, 2012; More et al., 2019). From the data available, this analysis could only be conducted for acute contact toxicity binary mixtures since no matching datasets for compound B were available for acute and chronic oral toxicity studies (Table S13–S15). OpenFoodTox and other databases (e.g. US-EPA, OECD e-chem portal, PPDB-Pesticide Properties Database, literature) provided 85% and 15% of values for compound B respectively. This approach is first described for available classes of chemicals namely a. insecticides-P-450 inhibitors and synergists, b. acaricides and insecticides, c. whole database. All individual binary mixtures data and summary statistics are available in supplementary material (Tables S1–S3, S13 and S15).

a) Insecticides-P-450 inhibitors (conazole fungicides, synergists)

Binary mixture experiments between insecticides and P450 inhibitors (e.g. conazole fungicides or synergists such as piperonyl butoxide - PBO) were the most investigated (55%) (Iwasa et al., 2004; Biddinger et al., 2013; Johnson et al., 2013; Spurgeon et al., 2016). For insecticides-conazole fungicides, the largest EMR values were observed for the pyrethroid insecticide tau-fluvalinate with prochloraz (≈1980

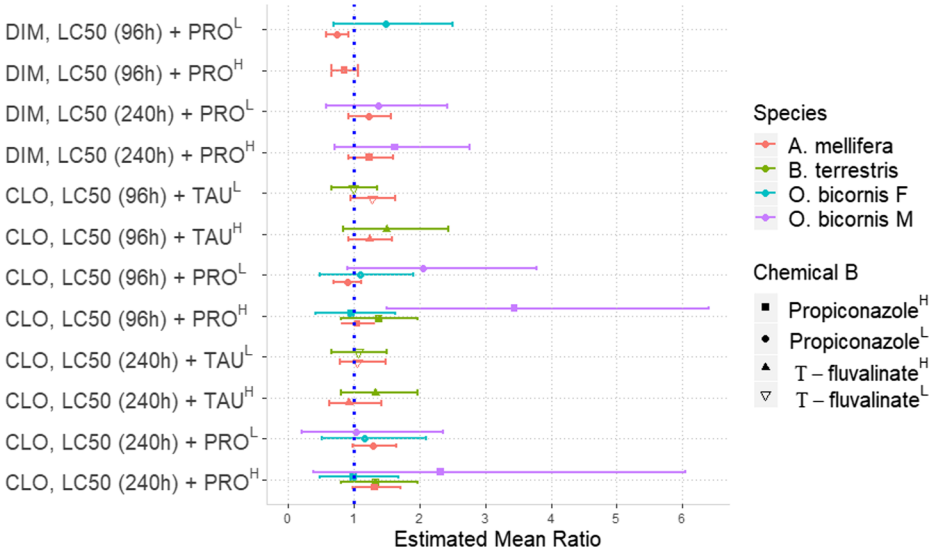

Fig. 2. Estimated Mean Ratio (EMR) following chronic oral exposure to binary mixtures in different bee species i.e. *A. mellifera*, *B. terrestris*, *and O. bicornis* female (F) or male (M). EMRs (dots) and related 95% CI (lines) were reported with different shapes according to the chemical B (see legend) investigated in the assay (Spurgeon et al., 2016; Robinson et al., 2017). Chemicals are reported as follows: CLO = Clothianidin; DIM = Dimethoate; PRO = Propiconazole; TAU = Tau-fluvalinate. Dose of chemical B is reported according to the author "high" (H) or "low" (L).

with TU_B = 0.07), thiacloprid-triflumizole (EMR ≈ 1460, TU_B = 0.50), neonicotinoids acetamiprid and thiacloprid with propiconazole (EMR of 101 and 490, respectively with TU_B = 0.07) (Johnson et al., 2013, 2012; Iwasa et al., 2004) (Table S3; Fig. 3). Although prochloraz had the lowest concentration within the mixture, it showed the highest synergistic effects (MDR = 20) when combined with tau-fluvalinate (LD_{50} = 19.8 µg/bee) (Fig. 3). In contrast, very low doses of azole fungicides showed a slight antagonist effect of 1.5-fold on pyrethroids (tau-fluvalinate with propiconazole TU_B = 0.0003 or myclobutanil TU_B = 0.001) (Johnson et al., 2013) (Table S3; Fig. 3). In addition, dose response data for the combined toxicity of tau-fluvalinate with myclobutanil (TU_B = 0.001, 0.01 and 0.07) and propiconazole (TU_B = 0.0003, 0.003 and 0.03) are illustrated following TU_B variation (Fig. 3).

Insecticides and synergists such as tau-fluvalinate-PBO showed the highest EMR (≈1980) and MDR (≈32), thus demonstrating strong synergistic effects even at low doses of PBO (TU_B = 0.03) (Fig. 4; Tables S3 and S15). EMRs for tau-fluvalinate, lambda-cyhalothrin and cyfluthrin with PBO at higher dose (TU_B = 0.34) were also large and very significant (≈ 945, 78 and 30 respectively) (Table S3 and S15; Fig. 4). It is interesting to note that when the three pyrethroids were tested without PBO, cyfluthrin shows the highest toxicity (LD_{50} 0.062 µg/bee) whereas tau-fluvalinate the least (LD_{50} 9.45 µg/bee) (Table S3). Our results confirm that the differential synergistic effects observed amongst the three pyrethroids is likely to be due to esterases acting on the acid moiety (Johnson et al., 2006). Indeed, tau-fluvalinate has an aromatic acid group, so that it is not sequestered as readily as the other pyrethroids and shows the greatest magnitude of synergism (Moores et al., 2012; Gunning et al., 2007). Lower magnitude of interactions were shown for Cyfluthrin (EMR = 2.3) and S,S,S-tributyl phosphorotrithioate (DEF) (EMR = 30) with PBO (TU_B = 0.34) (Table S3 and S15). Similarly, combined toxicity of lambda-cyhalothrin with diethyl maleate (DEM) (EMR ≈ 3) and PBO (TU_B = 0.34) (EMR ≈ 80) indicated greater synergism in the presence of PBO. The scientific basis for such interactions is of metabolic nature since PBO is a potent CYP inhibitor and DEF inhibits carboxylesterases (Johnson et al., 2013; Johnson, 2015; Mao et al., 2017; Wu et al., 2007).

Overall, hymenoptera are known to have a specific metabolic profile with the lowest copy number of detoxification enzymes within the insect kingdom (Johnson et al., 2013, 2015; EFSA, 2013a). In particular, honey bees have one of the lowest numbers of CYP genes isoforms of any invertebrate sequenced (46 sequences). Therefore, our results confirm that sterol biosynthesis–inhibiting (SBI) fungicides inhibit the CYP-mediated detoxification of some pyrethroids (e.g. tau-fluvalinate) and neonicotinoids (e.g. imidacloprid), thus increasing the acaricide and insecticide toxicity to bees, respectively (Wade et al., 2019; Pilling et al., 1995; Iwasa et al., 2004; Johnson et al., 2013).

b) Acaricides-Insecticides

Combined toxicity was synergistic for tau-fluvalinate and coumaphos in a dose dependent fashion (TU_B = 0.005, 0.01, 0.05, 0.15, 0.49) with the highest EMR ≈ 30 (TU_B = 0.49). In contrast, the magnitude of synergism between coumaphos and tau-fluvalinate (TU_B = 0.01, 0.03, 0.08, 0.25) reached a maximum EMR of 3-fold at the highest doses (TU_B = 0.08, 0.25). Both compounds are known CYP inhibitors but based on the limited data available for these two binary mixtures further dose response data would be needed to better characterise the dose dependency of such interactions (Hesketh et al., 2016). In addition, both tau-fluvalinate and coumaphos are lipophilic and are absorbed by the wax component of the hive, thus persistent after repeated treatments and these aspects should be taken into account under field scenarios (EFSA PPR Panel, 2012). In addition, temporal transitivity (i.e. if the same effect occurs irrespective of the order of exposure) of the interactions should be taken into account when assessing acaricide-insecticide mixtures: fenpyroximate pre-treatment (TU_B = 0.06) increased tau-fluvalinate toxicity by 8 fold (MDR = 5.56), whereas the opposite is not observed (EMR = 1.2) thus showing additive effects (MDR = 1.09) (Fig. 5; Table S15). Apparently, fenpyroximate can competitively inhibit CYP isoforms involved in tau-fluvalinate detoxification while tau-fluvalinate does not interact with CYPs, thus allowing bees to tolerate fenpyroximate exposure (Mao et al., 2011; Johnson et al., 2013) (see Fig. 5).

Experimental studies on combined toxicity (LD_{50}) following acute contact exposure to binary mixtures (PPPs - synergists) in different bee subspecies is presented in Fig. 6 (Rinkevich et al., 2015). Results show that bioassays using amitraz (acaricide), coumaphos (insecticide) and piperonyl butoxide (P450 inhibitor) increase phenothrin (insecticide) acute contact toxicity in all three different honey bee subspecies (i.e. Carniolan, Italian, and Russian bees). However, with regard to phenothrin sensitivity test (Fig. 6) between the three different honey bees subspecies, toxicity increased by a maximum of 7 fold in *A. mellifera*

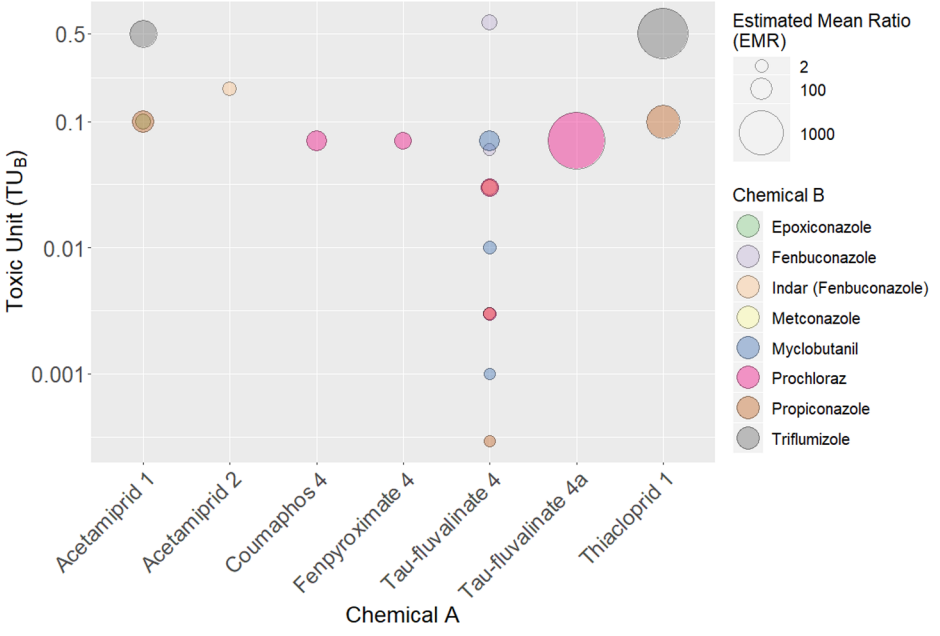

Fig. 3. Bubble plot for acute contact toxicity of insecticides (chemical A) and conazole fungicides (chemical B) in honey bees: Estimated Mean Ratios (EMR) (A + B) and experimental potency-adjusted dose (chemical B: Toxic Unit - TU_B). Size of the bubble is proportional to the value of the EMR. Colours represent different chemicals as reported in the legend. 1 = Iwasa et al. (2014). 2 = Biddinger et al. (2013). 4, 4a = Johnson et al. (2013). All the studies were statistically significant according to non-overlapping 95% confidence intervals.

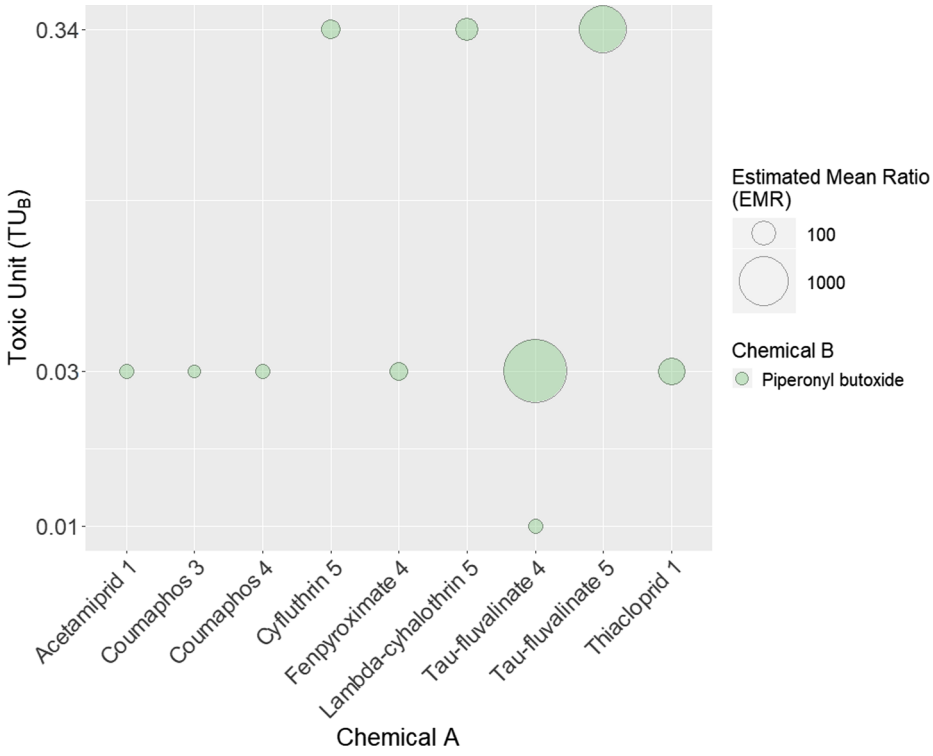

Fig. 4. Bubble plot for acute contact toxicity of insecticides (chemical A) and synergists (PBO) (chemical B) in honey bees: Estimated Mean Ratio (EMR) (A + B) and experimental potency-adjusted dose (chemical B: Toxic Unit (TU_B). Size of the bubble is proportional to the value of the EMR. 1 = Iwasa et al. (2014). 3 = Johnson et al. (2009). 4 = Johnson et al. (2013). 5 = Johnson et al. (2006). All the studies were statistically significant according to non-overlapping 95% confidence intervals.

primorski down to 5 fold in *A. mellifera ligustica* following acute contact exposure to coumaphos (Rinkevich et al., 2015).

c) Whole database

Figs. 7–9 compare EMRs for acute contact toxicity studies with their corresponding individual TU for compound B (TU_B) classified according to three different classes: $TU_B \leq 0.10$ (Figs. 7 and 8), $TU_B \leq 0.11$–0.30 and $TU_B \leq 0.31$–0.60 (Fig. 9). For each TU_B class, cumulative frequency distribution graphs are developed in order to quantify the sensitivity of the toxicological endpoint for chemical B contributing to the overall binary mixtures toxicity. The distribution of the EMR values against their "reverse cumulative frequency" is plotted and fits are tested with Pearson product-moment correlation coefficient (R^2). Results show that TU_B values range from 0.0001 to 0.61 (Table S15). Particularly, 63 (of out 133) binary mixtures experiments reported acute contact toxicity report TU_B values ≤ 0.1 (Figs. 7 and 8). This indicates that most of the doses of chemical B applied in the binary mixtures assay correspond to less than 10% of their estimated relevant critical endpoint (e.g. LD_{50} or LC_{50}). Furthermore, if looking at EMRs, the highest binary mixture toxicity (EMR ≈ 1980) is obtained when low doses of chemical B is applied in the binary mixture (i.e. $TU_B = 0.03$) (Fig. 8; Table S15). Hence, our findings would raise a concern that mixtures of contaminants, although individually at low concentrations

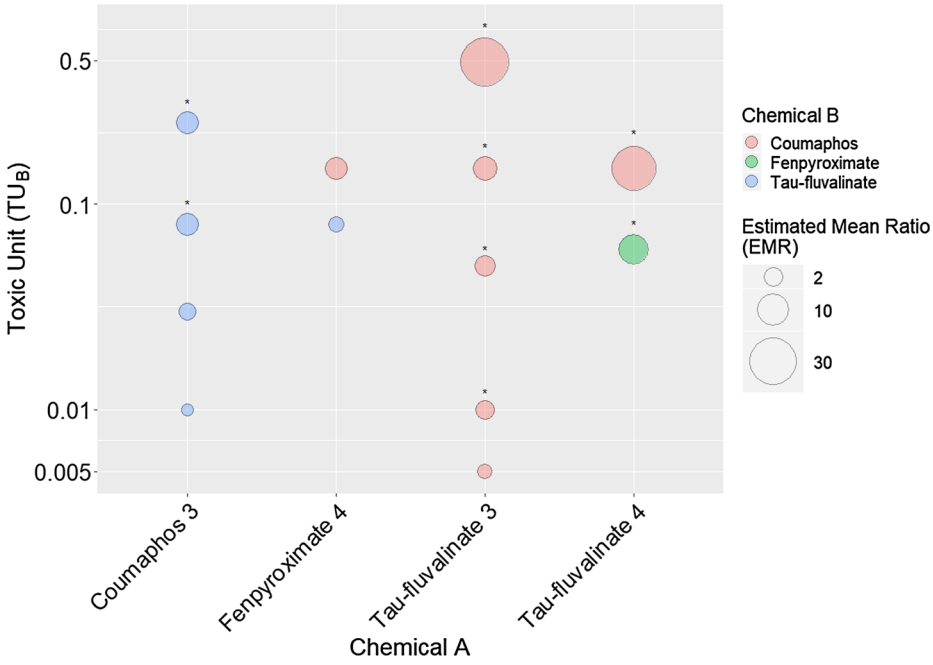

Fig. 5. Bubble plot for combined acute contact toxicity of acaricides (chemical A) and insecticides (chemical B) in honey bees. Estimated Mean Ratios (A + B) and experimental potency-adjusted dose (chemical B: Toxic Unit (TUB). Size of the bubble is proportional to the value of the EMR. References: 3 = Johnson et al. (2009). 4 = Johnson et al. (2013). * = for statistically significant studies.

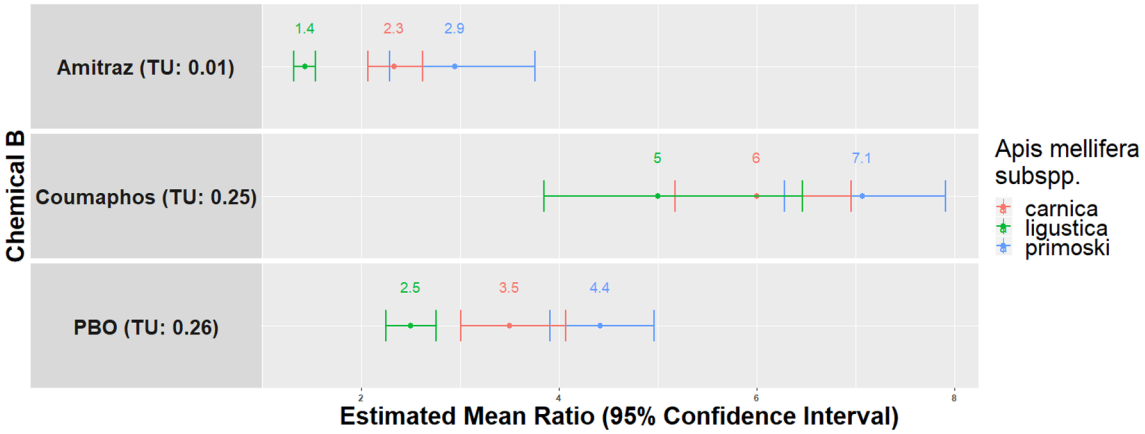

Fig. 6. Fore stplot comparing honey-bee subspecies sensitivity to combined toxicity of binary mixtures (Rinkevich et al., 2015). Estimated Mean Ratio (EMR dots) and related 95% CI (lines) were reported in different honey bee subspecies (*A. mellifera carnica, A. mellifera ligustica, A. mellifera primorski*) following acute contact exposure to phenotrin with three different chemicals (amitraz, coumaphos and PBO).

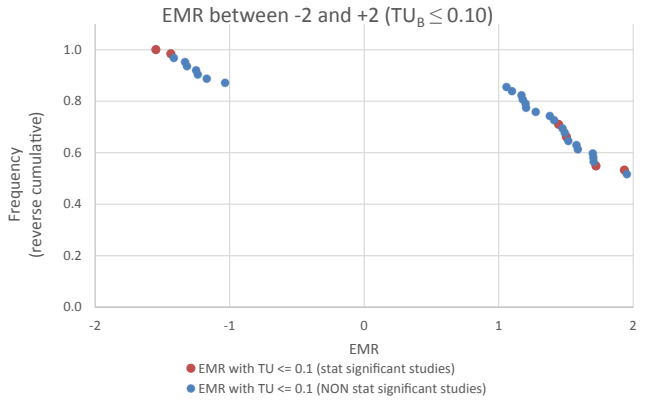

Fig. 7. Cumulative frequency distribution for Estimated Mean Ratio (EMR) values (EMR −2 - +2), in acute contact toxicity studies in honey bees reporting Toxic Unit for chemical B (TU_B) ≤ 0.10.

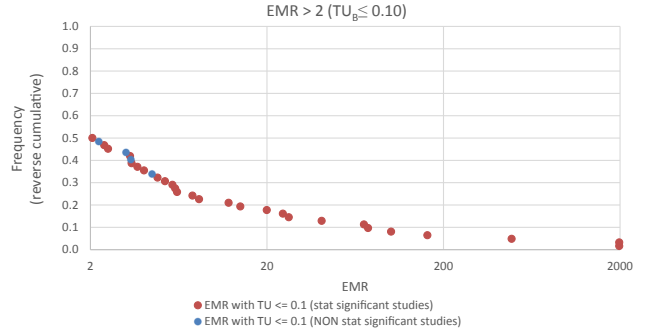

Fig. 8. Cumulative frequency distribution for Estimated Mean Ratio (EMR) values > (+)2, in acute contact toxicity studies in honey bees showing Toxic Unit for the chemical B (TU_B) ≤ 0.10.

(TU_B < 0.05), frequently may enhance the whole binary mixture toxicity (Cedergreen, 2014; Belden et al., 2007). However, it should be noted that at very low dose of chemical B (i.e. TU_B = 0.0003) a decrease mixture toxicity i.e. EMR (–) = 1.55 was observed (Fig. 7, table S15).

Overall, our results confirm that the observed synergism of binary mixtures in bees is, in most instances, explained as the result of toxicokinetic interactions at the level of metabolism either through the inhibition of a CYP or a transporter which then has toxicodynamic consequences i.e. pyrethroids and CYP inhibitor piperonyl butoxide,

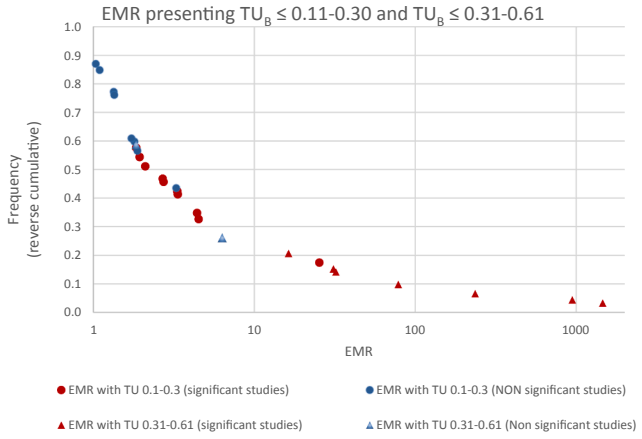

Fig. 9. Cumulative frequency distribution for Estimated Mean Ratio (EMR) values > 2, in acute contact toxicity studies in honey bees showing Toxic Unit for the chemical B (TU_B) > 0.10. TU values are split into two classes as provided in the legend. Dots represent TU values ≤ 0.11–0.30. Triangles represent TU ≤ 0.31–0.61.

insecticides with fungicides (Johnson et al., 2010; Moores et al., 2012). Generally speaking, toxicokinetic interactions of a mixture may cause deviations from additivity between components of the mixture either during absorption, distribution, metabolism or excretion.

3.2.3. Predictive models for combined acute contact toxicity and model deviation ratios

Comparison between predictive models of combined toxicity, to quantify the deviation from dose addition through the calculation of MDR values, are illustrated in Figs. 10 and 11 (see also Table S15) for the 92 acute contact toxicity binary mixtures (LD_{50} 24 h) in honey bees with available experimental dose response data (Jonker et al., 2005). For the oral route, chronic binary mixtures and wild bee species, no data were available to conduct this analysis. Hence, for this analysis, individual TUs for each compound in the binary mixture experiment were added to calculate the observed TU of the mixture (TUm) assuming CA as default model.

As described in Section 2.2.4, MDR values were calculated according to Belden et al. (2007). However, in our analysis, we proposed refined MDR thresholds in order to provide more conservative predictions for quantifying deviations from the CA model (Table 4). According to our MDR thresholds, from 92 binary mixtures of pesticides, combined toxicity of the binary mixtures was synergistic in 72% (66 datasets with 48 statistically significant), 17% additive (16 datasets) and

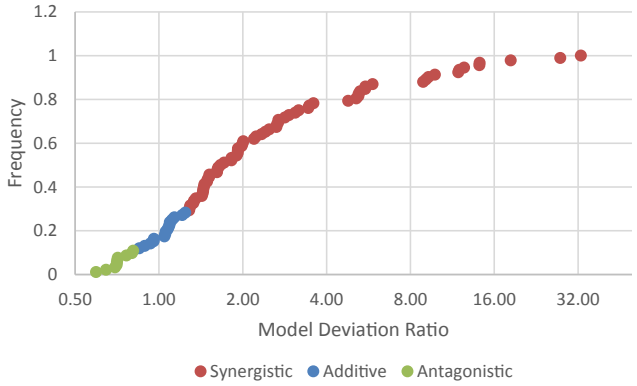

Fig. 10. Cumulated frequency of Model Deviation Ratio. MDR for acute contact toxicity studies resulting from the meta-analysis of acute contact toxicity studies on honey bees (Iwasa et al., 2004; Johnson et al., 2013, 2006, 2009; Ellis et al., 1997). MDR > 1.2 represents "synergistic" interactions, 0.83 < MDR < 1.25 represents "additive" effects; MDR < 0.83 represents "antagonistic" interactions.

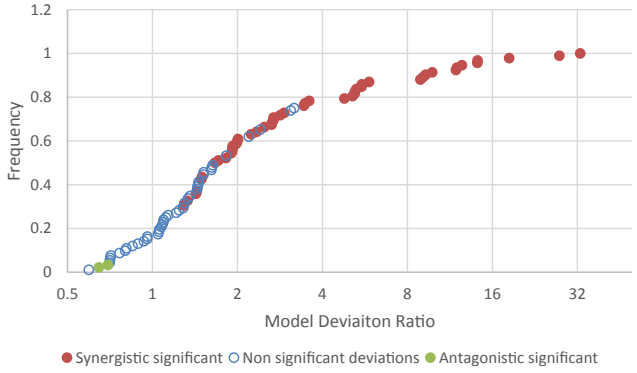

Fig. 11. Cumulated frequency of Model Deviation Ratio. MDR for statistically significant studies resulting from the meta-analysis of acute contact toxicity studies on honey bees (Iwasa et al., 2004; Johnson et al., 2013, 2006, 2009; Ellis et al., 1997). MDR > 1.25 represents "synergistic" interactions, 0.83 < MDR < 1.25 represents "additive" effects; MDR < 0.83 represents "antagonistic" interactions.

11% antagonistic (10 datasets with 2 statistically significant) (Table S15 and Figs. 10 and 11). Amongst synergies, the most commonly tested binary mixtures were conazole fungicide-insecticides combinations (Table S15). The statistical significance analysis for each binary mixture was performed using non-overlapping 95% CI of the experimental EM_A vs 95% CI of the experimental EM_M for chemical A + B). 16 out 66 mixtures were classified as statistical synergism according to our MDR thresholds (Table S15) although these were below the generic 2-fold deviation set as generic value by other authors regardless of target organism (e.g. *Daphnia* spp., honey bee), mixture (e.g. metals vs pesticides), exposure route (e.g. oral vs contact) and effects measured (e.g. lethal vs sublethal) in the experimental assays (Belden et al., 2007; Cedergreen, 2014). Here, we propose refined MDR thresholds to predict potential deviations from the DA model for the specific assessment of acute contact toxicity studies in honey bees.

3.2.4. Comparison of estimated mean ratios and model deviation ratios

Correlations between the analyses of EMR (3.2.2) and MDR predictions (3.2.3) for acute contact toxicity of binary mixtures in honey bees (n = 92) are presented on a scatterplot and Pearson product-moment correlation coefficient (R^2) in Fig. 12 (see also Table S15). Correlations between EMR and MDR values showed different reliability according to the type of experiment. In fact, when considering binary mixtures for compounds used in potentiation experiments (i.e. synergists, thus presenting $TU_B < 0.05$), the correlation between the two variables was highly reliable ($R^2 = 1$) (Fig. 12 - red dots). In contrast, for non-potentiation experiments ($TU_B > 0.05$) the correlation between the EMR and MDR slightly decreased ($R^2 = 0.72$). However, potentiation experiments of binary mixtures in honey bees are often reported as they reflect exposure to mixtures under field scenarios (Iwasa et al., 2004; Johnson et al., 2012, 2013; Cedergreen, 2014; Spurgeon et al., 2016; Robinson et al., 2017). Hence, EMR analyses can provide a reliable tool to predict combined toxicity of binary mixtures, conducted as potentiation experiments, given the toxicity of chemical A (LD_{50} A) and the binary mixture (LD_{50} A + B). This tool can be potentially useful when dose response data are scarce and do not allow an MDR analysis to be performed, particularly for the identification of mixtures which cause synergistic interactions in honey bees. However, limitations of the EMR approach have to be acknowledged since it does not fully comply with the DA principles and does not assume any mathematical model for the prediction of combined toxicity (DA, Response Addition, etc.).

The following thresholds for the EMR analysis are proposed:

- EMR < 0.95 indicates "antagonism" (i.e. corresponding to MDR < 0.83)
- 0.95 < EMR < 1.40 indicates "dose addition" (i.e. corresponding to 0.83 < MDR < 1.25)
- EMR > 1.40 indicates "synergism" (i.e. corresponding to MDR > 1.25)

4. Conclusions and implications for risk assessment

This manuscript constitutes the first consolidated **quantitative review of the available *in vivo* laboratory experiments on combined toxicity of binary mixtures in bee species** to support of hazard assessment. As noted in the introduction, exposure assessment and full risk characterisation are beyond the scope of this paper but their high relevance and implications for risk assessment are highlighted below together with future perspectives. Overall, 218 datasets were analysed with 61%, 20% and 19% reporting acute contact toxicity, chronic oral toxicity and acute oral toxicity respectively. Magnitude of interactions were estimated using EMRs, from experimental studies lacking dose response data (133 acute contact, 54 chronic oral and 41 acute oral datasets). Available dose response data for 92 binary mixtures (acute contact data) allowed the quantification of TU values, the testing of deviation from dose addition and the estimation of MDRs. Overall, dose addition, synergism and antagonism were found in 17%, 72% and 11% respectively.

Strong correlations were found between EMRs and MDRs particularly for experimental studies involving potentiation experiments indicating toxicokinetic (TK) interactions as **key mechanisms** through

Table 4
Comparison of Model Deviation Ratios (MDR) thresholds according to current scientific literature (Belden et al., 2007; Cedergreen, 2004) and refined MDR thresholds according to our analysis.

Mixture effect	Thresholds for Model Deviation Ratio (MDR) (according to Belden et al., 2007; Cedergreen, 2014)	Refined thresholds for Model Deviation Ratio (MDR)
Additive	0.5 ≤ MDR ≤ 2.0	0.83 ≤ MDR ≤ 1.25
Synergism	MDR > 2.0	MDR > 1.25
Antagonism	MDR < 0.5	MDR < 0.83

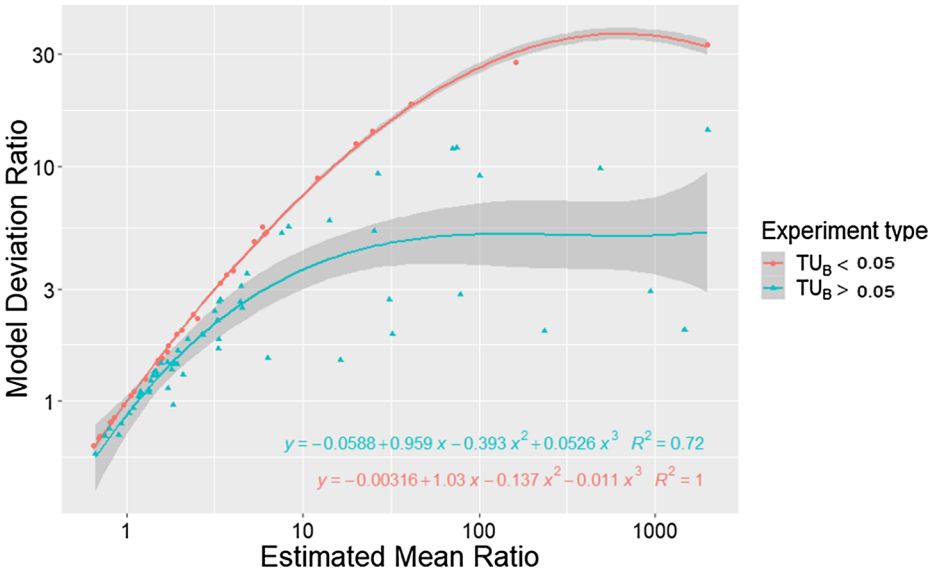

Fig. 12. Scatter plot investigating the correlation between Estimated Mean Ratio (EMR) and Model Deviation Ratio (MDR) for acute contact toxicity of binary mixtures in honey bees. Red dots represent potentiation experiments ($TU_B < 0.05$). Blue triangles represent no-potentiation experiments ($TU_B > 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mostly inhibition of metabolism (CYP, esterases and transporters) as demonstrated with the potent CYP inhibitors piperonyl butoxide, triazole fungicides, tau-fluvalinate, the carboxyl esterase inhibitor DEF and the transporter inhibitor ivermectin (Johnson et al., 2013, 2015; Guseman et al., 2016; Mao et al., 2017; Wu et al., 2007). In addition, bees have also been shown to have the lowest copy number of detoxification enzymes within the insect kingdom, particularly for CYP isoforms, methyltransferases and glutathione-s-transferases and inhibition of such limited metabolic capacity may also potentially lead to an increase in combined toxicity of chemicals (Johnson et al., 2013, 2015; EFSA, 2013a; Wade et al., 2019). Examples include inhibition of CYP-mediated detoxification by fungicides after exposure to the pyrethroid tau-fluvalinate or induction of imidacloprid metabolism leading to an increase in toxicity (Wade et al., 2019; Pilling et al., 1995; Iwasa et al., 2004; Johnson et al., 2013; Manning et al., 2017). A recent *in silico* docking study (Mao et al., 2017) using the active pocket of CYP9Q1, a broadly substrate-specific CYP with high quercetin-metabolising activity in the honey bee, and 121 pesticides, showed that six triazole fungicides inhibited CYP9Q1 though binding its catalytic site. In addition, five of six mitochondrial-related nuclear genes were down-regulated in adult honey bees fed binary mixtures of quercetin and the triazole myclobutanil and midgut metabolism of quercetin was reduced and was associated with reduced production of thoracic ATP, the energy source for flight muscles. Such findings have implications and authors conluded that, although fungicides have low acute toxicity, CYP inhibition interfering with quercetin detoxification may compromise mitochondrial regeneration, ATP production and bee health (Mao et al., 2017).

From this analysis, **key conclusions with regards to hazard assessment** of mixtures in bee species can be formulated:

1. Understanding the mechanistic basis of combined toxicity in bee species is critical for hazard assessment particularly because inhibition or induction of metabolism/transport may increase or decrease toxicity depending on the consequence of metabolism i.e bioactivation to a toxic metabolite or detoxification (Spurgeon et al., 2016; Hesketh et al., 2016; Guseman et al., 2016; Mao et al., 2017).
2. Available data on binary mixtures were mostly generated with well known inhibitors and may give a biased view of a more complex situation.
3. Applications of this analysis include: (a) use of the current open source database (DOI: https://doi.org/10.5281/zenodo.3383713) to provide scientific evidence for interactions and their magnitude for estimating mixture uncertainty factors for specific pesticide binary mixtures (see MIXTOX EFSA guidance (More et al., 2019)). (b) Development of *in silico* tools such as Quantitative-structure activity relationship (QSAR) models to predict combined toxicity of mixtures in honey bees for acute contact toxicity and other endpoints (chronic, sub-lethal), bee species (solitary bees, bumble bees) and routes (oral) in the future. Such models have been developed as classifiers for pesticides of different potency/threshold classes in bacteria (Toropova et al., 2012).
4. Key data gaps have been identified and include the need for: a) further laboratory testing and *in silico* docking studies in honey bees and wild bees to broaden our understanding of acute and chronic combined toxicity (contact and oral) and its dose dependency for different classes of pesticides and contaminants. This would support the characterisation of the synergistic potential of chemicals in bees including TK interactions either through inhibition or induction of metabolism or through direct toxicodynamic (TD) interactions. It is noted that chemical adjuvants and additives applied in pesticide commercial formulations may have a significant influence on combined toxicity and such formulations should be also tested either a components or as whole mixtures. b) Generation of basic TK (e.g. half life) and bioaccumulation data for chemicals in bee species to allow for the development and use of Dynamic Energy Budget (DEB) models for hazard assessment of mixtures in bee species (EFSA, 2013b; EFSA, 2014a; Hesketh et al., 2016; David et al., 2016; Rortais et al., 2017; EFSA, 2017a,b; Gradish et al., 2019).

Despite the above mentioned data gaps, availability of quantitative hazard metrics for combined toxicity will only provide a piece of the puzzle. Therefore, **addressing the exposure dimension** remains critical for (a) characterising the likelihood of co-occurrence of binary mixtures (or more complex mixtures) and (b) the potential magnitude of interactions at field relevant concentrations. Future directions to advance address exposure assessment science for honey bees and solitary bees include:

1. Data collection of realistic co-occurrence of multiple pesticide, veterinary drugs and contaminant residues in crops and plants visited by bees and bee matrices bearing in mind space and time,
2. Estimations of consumption data (e.g. contaminated sources such as nectar/pollen/water) for each bee species and life-stage (Tosi et al., 2018; EFSA AHAW Panel, 2016). This is particularly relevant for honey bees and wild bees (solitary and bumble bees) which can be exposed (via contact or oral routes) over a period of time, either directly through applications of multiple active ingredients in the

field or indirectly through consumption of contaminated pollen or nectar (Tosi et al., 2018; Johnson, 2015; EFSA, 2013a; Simon-Delso et al., 2017; Prado et al., 2019).
3. Exposure assessment of multiple pesticides and contaminants for different routes (aerial, chemigation or ground application) and over different seasons in the same crop as tank mixtures (Tosi et al., 2018).

For **risk characterisation**, a key recommendation for mixture assessment is the development of common risk metrics for honey bees and wild bee species which can then be compared to protection goals defined by risk managers. The choice of these methods is part of the iteration process of a fit for purpose mixture risk assessment which initiates in the problem formulation as part of the constant dialogue between risk assessors and risk managers (More et al., 2019). In principle, the risk metrics are selected using tiering principles depending on (a) context of the risk assessment (regulated products, contaminants, bee species and level of biological organisation (individual, hive, colony, population, landscape), (b) data available on exposure (co-occurrence at field relevant concentrations, consumption patterns, routes of exposure) and hazard (evidence for combined toxicity (dose addition, toxicokinetic interactions (e.g. synergism), bioaccumulation, timelines and resources (More et al., 2019). In such contexts, harmonised risk metrics can be developed and will be dependent on data gaps identified in this manuscript for the hazard and exposure dimensions ranging from low tier to high tier approaches. Low tier approaches include the application of the sum of TU i.e. individual TUs from laboratory LD_{50}s assuming dose addition and simple exposure estimates (e.g. rates of application of chemicals (e.g. pesticides) and default consumption in bees). High tier approaches can include probabilistic risk distributions for individuals, colony and population level based on the integration of model deviation ratios adjusted for internal dose (lethal or sub-lethal) using DEB models and probabilistic exposure assessment (co-occurrence, multiple routes, probabilistic consumption). At the population and species level, Species Sensitivity Distributions (SSDs) can also be applied to identify hazard concentrations (HCx) for multiple chemicals of concern according to the protection goal and compared to exposure estimates in populations (More et al., 2019). Low or high tier risk metrics are then compared to a given protection goal. The assessment may stops, if no concerns are identified. In contrast, indication of a potential risk for bee health may result in the need for a risk management decision or refinement of the risk characterisation using higher tier risk metrics (More et al., 2019).

Besides combined toxicity of multiple chemicals, a growing body of evidence has been published with regards to interactions between honey bee infectious agents (fungi, bacteria and viruses), predators, chemicals such as pesticides and contaminants (Collison et al., 2016; Hesketh et al., 2016), temperature and nutritional stressors (Tosi et al., 2017; Rortais et al., 2017). Examples provided in Table S10 include 1. combined exposure to clothianidin and imidacloprid and enhanced susceptibility of honey bees to deformed wing virus (DWV) (Di Prisco et al., 2013); 2. combined exposure toimidacloprid and *Nosema ceranae* (microsporidian parasite) in bees and increased sub-lethal effects and individual mortality rates (Alaux et al., 2010; Vidau et al., 2011; Pettis et al., 2012). 3. combined exposure to clothianidin, thiamethoxam and nutritional stress reducing honey bee survival (Tosi et al., 2017).

In order to take into account such complex stressors on bee health, the scientific Committee of EFSA is currently developing holistic approaches for the **risk assessment of multiple stressors in honey bees** at the individual, hive level, colony, population and landscape level from a request of the European Parliament (EFSA Scientific Commitee, in preparation). Key challenges for implementing such harmonised methods into practice, need to be highlighted with particular reference to key data gaps in bees: combined toxicity (lethal and sub-lethal, TK data), occurrence and consumption patterns, the need to develop common risk metrics (e.g. toxic units, risk ratios, margin of exposure) while applying tiering principles depending on context of the assessment, data available, timelines and resources (More et al., 2019). Finally, data from OMICs technologies can provide inputs to the honey bee colony model (APISRAM), under development at EFSA, to develop biormarkers of sub-lethal effects at the individual, hive, colony and population level and further quantify the impact of single and multiple stressors on bee health at the genome (transcriptomics), proteome (proteomics) and metabolome (metabolomics) level (EFSA, 2017a, b; Rortais et al., 2017; Aguilera et al., 2018).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105256.

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