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This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Fabio, S., Sonia, B., Teresa, R., Simone, T., Piotr, M., Roberto, M., et al. (2018). Lethal effects of Cr(III) alone and in combination with propiconazole and clothianidin in honey bees. CHEMOSPHERE, 191, 365-372 [10.1016/j.chemosphere.2017.10.068].

Availability:

This version is available at: <https://hdl.handle.net/11585/614400> since: 2018-01-04

Published:

DOI: <http://doi.org/10.1016/j.chemosphere.2017.10.068>

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(Article begins on next page)

This is the final peer-reviewed accepted manuscript of:

Fabio Sgolastra et al.

Lethal effects of Cr(III) alone and in combination with propiconazole and clothianidin in honey bees

which has been published in final form in *Chemosphere* Volume 191, January 2018, Pages 365-372

The final published version is available online at:

<https://doi.org/10.1016/j.chemosphere.2017.10.068>

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1 Lethal effects of Cr(III) alone and in combination with propiconazole and 2 clothianidin in honey bees

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15

16 Abstract

17 Several anthropogenic contaminants, including pesticides and heavy metals, can affect honey bee
18 health. The effects of mixtures of heavy metals and pesticides are rarely studied in bees, even
19 though bees are likely to be exposed to these contaminants in both agricultural and urban
20 environments. In this study, the lethal toxicity of Cr alone and in combination with the
21 neonicotinoid insecticide clothianidin and the ergosterol-biosynthesis-inhibiting fungicide
22 propiconazole was assessed in *Apis mellifera* adults. The LD₅₀ and lowest benchmark dose of Cr as
23 Cr(NO₃)₃, revealed a low acute oral toxicity on honey bee foragers (2049 and 379 mg L⁻¹,
24 respectively) and the Cr retention (i.e. bee ability to retain the heavy metal in the body) was

generally low compared to other metals. A modified method based on the binomial proportion test was developed to analyze synergistic and antagonistic interactions between the three tested contaminants. The combination of an ecologically-relevant field concentration of chromium with clothianidin and propiconazole did not increase bee mortality. On the contrary, the presence of Cr in mixture with propiconazole elicited a slight antagonistic effect.

30

Highlights

- Low acute oral toxicity of chromium on adults of honey bee foragers
- Chromium retention in bee body was 20-30% of the quantity ingested
- No synergistic effect between chromium and propiconazole or clothianidin
- Slight antagonism between chromium and propiconazole

36

Key words: heavy metals, pesticides, *Apis mellifera*, ecotoxicology, pollution, synergism/antagonism

39

1. Introduction

Bees are extremely important as crop pollinators and to maintain plant biodiversity (Klein et al., 2007; Ollerton et al., 2011). In the last decades, wild and managed bees have been declining worldwide thus posing a potential risk to food production and human health (Lautenbach et al., 2012; Chaplin-Kramer et al., 2014). Abnormal honey bee mortality rates have been observed in US and in European Countries, with percentages of overwintering colony losses much higher than 10% rate that is usually considered an acceptable loss threshold value by beekeepers (Lee et al., 2015; Chauzat et al., 2016). Many factors have been taken into account to explain this phenomenon (Biesmeijer et al., 2006; Potts et al., 2010; Abbo et al., 2017; Fauser-Misslin et al., 2014; Dance et al., 2017). Pesticides, malnutrition, pathogens (including *Varroa* mite infestation), climate change, habitat fragmentation and some beekeeping management practices (e.g. migration activities for almond pollination in US) are the main factors that affect honey bee survival (Goulson et al., 2015). However, up to now, these stressors have often been studied individually and the potential synergic effects of other anthropogenic activities, like heavy metal pollution, have rarely been considered.

In fact, although the use of honey bees as environmental bioindicator of heavy metals have been studying since 1935 (Svoboda, 1961), the effects of these pollutants on bee health have often been overlooked and only recently they are considered in the framework of bee decline (Morón et al., 2012; Exley et al., 2015).

In the present study, we addressed the lethal effects of chromium as Cr(III), alone and in combination with the neonicotinoid clothianidin and the ergosterol-biosynthesis-inhibitor (EBI) fungicide propiconazole on honey bees (*Apis mellifera ligustica* L.) following acute oral exposure under laboratory conditions. Chromium is a heavy metal ubiquitous in the environment often found as Cr (III) or (VI). The environmental diffusion of Cr has been increasing in the last years due to mining and industrial activities (Zayed and Terry, 2003). Although Cr(III) is commonly present in

64 animals, it becomes toxic at high concentrations (Di Bona et al., 2011). Since this metal may be
65 accumulated in plant tissues (Oliveira, 2012), honey bees can be exposed to this contaminant by
66 contact and ingestion. As a consequence, chromium can be found in honey (Conti and Botrè, 2001;
67 Porrini et al., 2002; Satta et al., 2012). Honey bees are considered bioindicator of environmental Cr
68 pollution since environmental levels detected in honey bee matrices (i.e. honey, bee body, beeswax)
69 range from 0.005 to 46.52 mg kg⁻¹ depending on the matrix considered or on environmental colony
70 location (i.e. rural, urban or industrial area) (Porrini et al., 2002; Satta et al., 2012).

71 LD₅₀ of heavy metals are rarely assessed in bee ecotoxicology (Hladun et al., 2013; Di et al. 2016;
72 Heard et al., 2017; Robinson et al., 2017) and no value is available in literature for Cr as well as its
73 benchmark dose (BMD) (*i.e.* the estimated lowest dose that produces an adverse response compared
74 to the negative control).

75 Clothianidin and propiconazole pesticides are commonly applied to various crops such as oilseed
76 rape, sunflower, fruit trees, maize and cereals (EFSA, 2013a; 2013b; Simon-Delso et al., 2015) and
77 their residues are often found in honey bee matrices (Lambert et al., 2013; Mullin et al., 2010;
78 Pistorius et al., 2015; Porrini et al., 2016). Therefore, the co-exposure of bees to these compounds
79 under field conditions should be investigated.

80 Previous studies have already reported that clothianidin and propiconazole may interact in a
81 synergistic way in honey bees following acute oral or contact exposure (Biddinger et al., 2013;
82 Thompson et al., 2014; Sgolastra et al., 2017). However, no information on possible interactions
83 among Cr and these two pesticides is available.

84 In this study, the LD₅₀ of Cr (expressed both in mg L⁻¹ sugar syrup and in µg bee⁻¹) and its BMD
85 (expressed in mg L⁻¹) at 48 hours after ingestion were determined for the first time. In addition,
86 possible lethal effects of environmental Cr concentrations in combinations with clothianidin and
87 propiconazole (i.e., binary or ternary mixtures) were investigated and a new statistical method to

88 define synergistic/antagonistic interaction among them was developed *ad hoc*. Finally, Cr
89 bioconcentration ratio in the bee body (i.e., bee Cr concentration/feeding solution Cr concentration),
90 as a measure of honey bee capacity to retain the heavy metal, was estimated.

91

92 **2. Materials and methods**

93 *2.1 Bees and test conditions*

94 Forager honey bees (*Apis mellifera ligustica*) were obtained from three healthy colonies placed in
95 an experimental apiary of CREA-AA (Bologna, Italy). During summer 2015, forager bees were
96 collected using the “Funnel trap” (Medrzycki, 2013). The trap placed at the entrance of the hive
97 allows collecting only forager bees, thus reducing the variability among bee categories (i.e., guard
98 and other in-hive bees). After 30 min of anesthetization with 60% CO₂ in synthetic air, bees were
99 placed in cardboard cages (9.5 cm x 6.5 cm x 5 cm) in groups of 10 (LD₅₀ and BMD estimations) or
100 20 individuals (single pollutants, binary/ternary mixtures exposure experiment) per cage. Three
101 cages per treatment were used. Bees from each colony were randomly distributed in group of 10 (or
102 20) among treatments to account for genetic diversity (i.e. different colony origin). In addition, to
103 exclude any potential colony effect, a rank-transformed repeated-measures ANOVA analyses
104 (Zimmerman and Zumbo, 1993) was performed for each treatment, with colony as the between-
105 subjects factor and time (4, 24, 48, 72 and 96 h) as the within-subjects factor. In all treatments, no
106 differences among colonies were found (Tables S1 and S2 in the Supplementary Information).
107 During the experiment, the cages were maintained at 25±2 °C and 50-70% of relative humidity in
108 an incubator under complete darkness. The cages were daily rotated to reduce potential differences
109 in the incubator microclimate.

110 All treatments were performed on bees after 1 h starvation period. Test solutions (*vide infra*) were
111 provided using a bulk feeder. For each treatment, the volume provided per cage was defined

112 according to the assumption that, through trophallaxis, all individuals would ingest similar doses of
113 10 μ L (OECD, 1998; Medrzycki et al., 2013). At the end of the exposure phase (maximum 2 h), the
114 complete consumption of the solution was verified by visual inspection of the feeder. After that,
115 bees were fed *ad libitum* with a sugarbeet (Eridania Italia SpA, Italy) syrup solution
116 (sugarbeet:distilled water = 50:50 w/v) until the end of the experiment (96 h). Dead bees were
117 preserved at -20 °C until elemental analysis.

118

119 2.2 Chemicals

120 $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (MW 400.15 g mol⁻¹) and $\text{Cr}_2(\text{SO}_4)_3$ (MW 392.18 g mol⁻¹) were purchased from
121 Carlo Erba (Italy).

122 Propiconazole with 98.4% purity and clothianidin with 99% purity were purchased from Sigma-
123 Aldrich (USA) and from Dr Ehrenstorfer GmbH (Germany), respectively. The main chemical
124 characteristics of the two pesticides are reported in Table 1.

125

126 2.3 Estimation of Cr(III) LD₅₀ and BMD

127 Bees were exposed to different doses of $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ in a geometric series in order to calculate
128 the dose-response curve and estimate the LD₅₀ and BMD of Cr. As defined by a range-finding test,
129 the following Cr concentrations in the sugar syrup solution (50% w/v) were chosen: 514, 1632,
130 2167, 2667 and 4605 mg Cr L⁻¹. Among treatments, the highest concentration (4605 mg Cr L⁻¹) was
131 excluded in the calculation of the dose-response curve because the solution was not completely
132 consumed by bees at the end of the exposure phase, likely due to its repellent effect. The toxicity of
133 Cr as $\text{Cr}_2(\text{SO}_4)_3$ was also tested at the Cr concentrations of 302, 932, 1336, 1865, and 2685 mg L⁻¹
134 to evaluate possible effect of the Cr counterion.

135 The Cr concentrations in the test solutions were determined by elemental analysis with an
136 inductively coupled plasma optical emission spectrometer (*vide infra*).

137 Control cages were supplied with sugar syrup solution.

138

139 2.4 Bee treatments with single component solutions, binary and ternary mixtures

140 A propiconazole solution at the concentration of 700 mg L^{-1} was prepared by dissolving 700 mg of
141 the fungicide in 15 mL of acetone (purity >99.0%, Sigma-Aldrich, USA) and then adding sugar
142 syrup solution (50:50 w/v) up to 1 L of final volume. Aliquots of $10 \text{ }\mu\text{L}$ of the solution containing 7
143 μg of propiconazole were provided per-capita to the bees: the dose was chosen as a non-lethal dose
144 as previously defined (Sgolastra et al., 2017). This dose corresponds at $\sim 1/9$ the oral LD_{50} at 24 h
145 for *Apis mellifera* (Ladurner et al., 2005).

146 A clothianidin solution at the concentration of 0.074 mg L^{-1} was prepared by dissolving 0.074 mg
147 of the insecticide in 15 mL of acetone and then adding the sugar syrup solution up to 1 L of final
148 volume. Solution aliquots of $10 \text{ }\mu\text{L}$ containing 0.74 ng of clothianidin were provided per-capita to
149 the bees: the dose falls within the range of the $\text{LD}_{10\pm 95\%}$ confidence limit (CL) for clothianidin in
150 *A. mellifera* as previously estimated (Sgolastra et al., 2017). This dose can be also considered
151 ecologically relevant since it is within the range of the estimated amount of clothianidin ingested by
152 a honey bee during a foraging bout (0.11-1.36 ng) (Sgolastra et al., 2017).

153 A sugar syrup solution (sugar:distilled water = 50:50 w/v), containing 1.5% of acetone and 3.9 mg
154 Cr L^{-1} as $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, was prepared for the evaluation of the effect of the environmental Cr
155 concentration on bees. Solution aliquots of $10 \text{ }\mu\text{L}$ containing $0.039 \text{ }\mu\text{g}$ of Cr were provided per-
156 capita to the bees. This concentration was chosen because it falls within the Cr concentrations found
157 in honey bee matrices (Porrini et al., 2002; Satta et al., 2012) and thus it can be considered
158 ecologically relevant.

159 Binary solutions were prepared by dissolving into 15 ml of acetone: i) 700 mg of propiconazole and
160 0.074 mg of clothianidin; ii) 700 mg of propiconazole and 3.9 mg of Cr as $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$; iii)
161 0.074 mg of clothianidin and 3.9 mg of Cr as $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$. All the organic solutions were then

162 diluted with sugar syrup solution up to 1 L of final volume. Aliquots of 10 μ L of each binary
163 solution were provided per-capita to the bees.

164 A ternary solution was prepared by adding to 1 L of the binary solution of propiconazole and
165 clothianidin, 3.9 mg of Cr(III) as $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$. Even in this case, aliquots of 10 μ L of the ternary
166 solution were provided per-capita to the bees.

167 Acetone (15 mL) was diluted to 1 L with the sugar syrup solution as a control (solvent control). In
168 addition, the syrup solution was also tested on bees as a negative control.

169

170 *2.5 Metal content analysis*

171 Metal concentrations in contaminated syrup solution and in honey bee body were measured after 48
172 h from exposure phase by using an inductively coupled plasma optical emission spectrometer (ICP-
173 OES) furnished by SPECTRO Analytical Instruments GmbH & Co. (Kleve, Germany) equipped
174 with a plasma source and an optical detector with a charge-coupled device (CCD) able to quantify
175 emission wavelengths of elements ranging between 125 and 780 nm. Test solutions were analyzed
176 for Cr after addition of HNO_3 ($\geq 69\%$ v/v, for trace analysis, Sigma-Aldrich, USA).

177 Single honey bees (mean \pm SE dry weight: 22.75 ± 0.47 mg each) were analyzed for Cr content after
178 dissolution in a mixture of HNO_3 ($\geq 69\%$ v/v, for trace analysis, Fluka, Sigma-Aldrich, USA) and
179 H_2O_2 (30% v/v, for trace analysis, VWR Prolabo Chemicals, USA) in the ratio of 4:1 (v:v) by
180 microwave-assisted digestion (*Start D*, Microwave Digestion System, Milestone, USA) before
181 elemental analysis. The limit of detection (LOD) for Cr was $0.38 \mu\text{g kg}^{-1}$ bee. For the statistical
182 analysis, zero value was assigned to concentrations below the limit of detection (*vide infra*).

183 The Cr recovery from bee matrix exposed to digestion and then analysed by ICP-OES was
184 determined as follows. After drying at 100°C for 24 h, five bees were singly spiked with 10 μ L of a
185 Cr standard solution ($1000 \text{ mg Cr L}^{-1}$) for ICP-OES calibration and additional five control bees
186 were added with the same volume of distilled water. Once the added solutions were reduced by
187 evaporation (within ca. 2 h), bees were singly mineralized and processed for Cr determination as

188 already described. Cr recovery on spiked bees resulted $102 \pm 1.6\%$ and Cr content of control bees
189 was always below the LOD.

190

191 2.6 Statistical analysis

192 The number of dead bees was measured 4, 24, 48, 72 and 96 h after exposure to pollutants (see
193 Figures S1 and S2 of Supplementary Information for mortality data vs time, corrected with Abbott's
194 formula for Cr as $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ or $\text{Cr}_2(\text{SO}_4)_3$). Both the BMD intervals (BMDL-BMDU) and
195 LD_{50} values of Cr were estimated at 48 h after exposure phase.

196 The LD_{50} s were estimated with a Probit analysis (Finney, 1952) at 95% CL. The values expressed
197 in mg Cr L^{-1} in the sugar syrup were then transformed in $\mu\text{g bee}^{-1}$ assuming that each bee ingested
198 10 μL of test solution.

199 The Cr BMD intervals were estimated using PROAST version 62.5 (<http://www.proast.nl>). The
200 BMD approach is considered as an alternative of the no-observed-adverse-effect level (NOAEL)
201 approach, since it makes a more extended use of available dose-response data and provides a
202 quantification of their uncertainties (EFSA, 2009). The approach considers the dose-response
203 information by fitting several mathematical models to the data. Our dose-response data were
204 analysed according to EFSA (EFSA, 2009, 2017). Briefly, the Bench Mark Response (BMR), also
205 known as Critical Effect Size, was set at 10% as recommended for quantal data analysis. The BMD
206 is the dose, derived from the estimated dose-response curve, associated with the BMR. The lower
207 and upper bounds of the BMD, denoted BMDL and BMDU, correspond to the projection of the
208 lower and upper 95% one sided confidence bound of BMR, respectively, to the dose axis. The
209 BMD intervals for each fitted model were reported following the EFSA recommendations (EFSA,
210 2017) so that the lowest BMDL and highest BMDU from these selected models were then used to
211 define the final BMD confidence interval.

212 The quantity of Cr retained by single bees (expressed in $\mu\text{g mg}^{-1}$ of dry body weight) and the metal
 213 bioconcentration ratio (MBR), i.e. the ratio between Cr ingested and Cr found in bee body, were
 214 evaluated with a regression analysis (see Section S3 and Figures S3 and S4 in Supplementary
 215 Information).

216 In the experiment where bees were exposed to pollutants as single compound or binary/ternary
 217 mixtures, Log-rank Kaplan-Meier (K-M) survival analyses with pairwise multi comparison
 218 procedures (Hom-Sidak method) were carried out to compare survival among treatments. Survival
 219 analyses were conducted with SigmaPlot 12.3.

220 For each assessment time (i.e. 4, 24, 48, 72 and 96 h after exposure to pollutants), the binomial
 221 proportion test described in Sgolastra et al. (2017) was used to estimate potential synergism on bee
 222 mortality between the different combinations of chromium and the two pesticides. In addition, the
 223 test was modified in order to assess antagonistic interactions. Since antagonism and synergism were
 224 tested on the same dataset and at five different times, we used a multiple comparison correction
 225 (Holm, 1979) to estimate significance levels for 10 p-values jointly. The null hypotheses that we
 226 were trying to test were:

$$H_0 \equiv p_{AB}^{obs} - p_{AB}^{exp} = p_{AB}^{obs} - (p_A + p_B - p_A \cdot p_B) > 0$$

227 when synergy was expected, and:

$$H_0 \equiv p_{AB}^{obs} - p_{AB}^{exp} = p_{AB}^{obs} - (p_A + p_B - p_A \cdot p_B) < 0$$

228 when antagonism was expected. According to Bliss independence criterion, the expected combined
 229 effect of two substances in an organism is expressed as follows:

$$p_{AB}^{exp} = p_A + p_B - p_A \cdot p_B$$

230 where p_A and p_B represent the mortality probability associated with the use of substances A and B,
231 respectively, and p_{AB}^{exp} is the expected mortality of their combined effect (see the R script at section
232 S4 in Supplementary Information).

233

234 3. Results and discussion

235 Although the co-exposure to heavy metals and pesticides can likely occur in agricultural and urban
236 environment, their effects in combination have been rarely evaluated in bees (Jumarie et al., 2017).
237 This study was aimed at assessing the lethal toxicity of Cr alone and in combination with two
238 common pesticides: the neonicotinoid insecticide clothianidin and the EBI fungicide propiconazole
239 under laboratory conditions. In general, results from laboratory studies are usually considered
240 conservative in risk assessment (worst case scenario) since chemicals are better protected by
241 environmental degradation (Cluzeau, 2002). In addition, data obtained in laboratory conditions are
242 more reliable and comparable because of the adopted standard methods. However, several
243 ecologically important effects (i.e. sublethal effects that can affect the whole colony) are difficult to
244 detect under the same conditions.

245

246 3.1 Chromium LD_{50} and BMD

247 The Cr LD_{50} and BMD intervals (BMDL and BMDU) estimated at 48 hours in the acute oral
248 toxicity tests are reported in Table 2.

249 The values of LD_{50} are expressed both as mg Cr L^{-1} sugar syrup and μg Cr bee $^{-1}$. For the LD_{50} , the
250 CL ranges obtained for Cr as $Cr(NO_3)_3$ is well overlapped with the range values obtained for Cr as
251 $Cr(SO_4)_3$, thus excluding possible lethal effects of Cr counterion. The calculated Cr LD_{50} in *A.*
252 *mellifera* adults equals to 2049 mg L^{-1} (or 20.5 μg bee $^{-1}$) which indicates slight toxicity based on the
253 WSDA pesticide's classification (WSDA, 2010), especially when compared to other pollutants

(e.g.: Se LD₅₀: 60 mg L⁻¹ (Hladun et al., 2013); Cu LD₅₀: 72 mg L⁻¹ and Pb LD₅₀: 345 mg L⁻¹ (Di et al., 2016); Cd LD₅₀: 18.36 mg L⁻¹ and As LD₅₀: 25.68 mg L⁻¹ (Heard et al., 2017)).

As far as the BMD is concerned, a detailed description of the BMD analysis according EFSA guideline (EFSA 2009; 2011) is reported in section S4 of Supplementary Information (Tables S6 and S7). According to this analysis, the lowest BMD limit determined for Cr as Cr(NO₃)₃ (BMDL: 379 mg Cr L⁻¹, Table 2) is one order of magnitude higher than the highest environmental concentrations found in honey bee matrices (46.52 mg Cr kg⁻¹, Satta et al., 2012). According to our data, Cr at environmental concentrations poses a relatively low risk to honey bee adults by acute oral exposure.

The effects of Cr have also been addressed in other insect species however it is very difficult to compare their results to our findings due to the relevant differences in the methodologies adopted. For example, several studies focused on Cr exposure during larval stage (*Drosophila melanogaster*: Hepburn et al., 2003; *Bombyx mori*: Tucker et al. 2003; *Galleria mellonella*: Wu and Yi, 2015; *Hermetia illucens*: Gao et al. 2017), others tested Cr(VI) (*Culex quinquefasciatus*: Sorensen et al. 2006; *Oxya chinensis*: Li et al. 2005) or assessed different endpoints (e.g. genotoxicity and reproduction in *D. melanogaster*: Hepburn et al., 2003). Finally, other studies dealt with aquatic insects with exposure via water environment (Warnik and Bell 1969; Rehwoldt et al. 1973).

3.2. Bioaccumulation of chromium in bee body

Figure 1 shows the Cr retained in bee body (a) and the MBR (b) as a function of Cr dissolved in the syrup ingested by the bees. No Cr residues were detected in control bees. Observational data in Figure 1a,b were fitted with statistical models (see section S3 in Supplementary Information) in order to model the dependence of Cr retained and MBR datasets on Cr dissolved in syrup.

277 The Cr-retained dataset showed a positive and very significant linear relationship with Cr in the
278 feeding solution ($p < 0.001$ for α_{Al} coefficient and $p = 0.0880$ for β_{Al} : Table S3 in Supplementary
279 Information).

280 On the other hand, the MBR data showed a weak increasing trend with Cr in syrup. A non-linear
281 curve constrained to pass through the origin of coordinates (see section S3.2 in Supplementary
282 Information) showed a good agreement with the observed MBR points, although its coefficients
283 were not statistically significant. Similar nonlinear MBR trends with the metal concentrations in the
284 syrup have been reported for Al, Pb and Cd in honey bee body following chronic exposure
285 (Gauthier et al., 2016). Remarkably, our data show that Cr accumulated in the bee body was 20-
286 30% of Cr ingested (0.2–0.3 MBR values) within the tested concentration range (514-2667 mg Cr
287 L^{-1}).

288 In our study, the Cr retention in bee body after acute exposure was generally lower than the values
289 observed after Al, Pb, Cd and Fe chronic exposure, thus suggesting bee higher ability to eliminate
290 Cr compared to other heavy metals (Gauthier et al., 2016; Jumarie et al., 2017). Seemingly, the low
291 toxicity of Cr in bee compared to other heavy metals (Hladun et al., 2013; Di et al., 2016; Heard et
292 al., 2017; Robinson et al., 2017) might be related to bee ability to eliminate the heavy metal from
293 the body.

294

295 3.3. Experiment with the mixtures of chromium, clothianidin and propiconazole

296 Cumulative proportion of surviving bees to Cr, propiconazole and clothianidin as single compounds
297 and as binary and ternary mixtures are presented in Figure 2. Significant differences among
298 cumulative survival curves of honey bees exposed to different treatments were found (Log-rank
299 analysis $\chi^2 = 87.6$, $df = 8$, $p < 0.001$). In order to better highlight differences among treatment effects on

bee mortality, pairwise analysis was performed on survival curves of Figure 2 and the p values are reported in Table 3.

In details, the clothianidin and propiconazole combination in the absence (CLO+PRO) or in the presence of Cr (CLO+PRO+Cr), as well as clothianidin and chromium mixture (CLO+Cr), gave the lowest bee survival after 96 hours from ingestion (Figure 2). As far as the bee survival within 4 days observation is concerned, no significant differences were observed among the combined treatments (i.e., CLO+PRO, CLO+PRO+Cr, CLO+Cr); however, the survival rates were significantly lower than controls (Table 3). On the contrary, after 96 hours from ingestion, bee exposure to single pollutants (i.e., PRO, CLO and Cr) or to propiconazole and Cr combination (PRO+Cr) resulted in a more limited mortality if compared to the other treatments (Figure 2). As reported in Table 3, no significant differences ($p>0.05$) were observed among survival curves of these treatments and the two controls (negative and solvent controls), thus confirming that our test doses were sublethal when administered alone.

In this study, the binomial proportion test developed for synergism (Sgolastra et al., 2017) was implemented to evaluate the antagonistic effect of the three pollutants in binary or ternary mixtures on bee mortality (Table 4). The script of this new procedure is provided as a Supporting data. Briefly, the implemented test is able to highlighten both the synergistic or antagonistic effect size expressed as a positive or negative difference, respectively, between the observed and expected mortality probabilities for each pollutants combination at each assessment time. In Table 4, A or B terms refer to the effect size of single pollutants in binary or in ternary mixture. The lethal effect on bees of clothianidin and propiconazole combination (A and B terms, respectively, Table 4) was synergistic for the first 48 hours after ingestion as shown by the significantly ($p<0.05$) positive values of effect size, in full agreement with previous results (Sgolastra et., 2017). The mechanism responsible for the synergism between the two pesticides is well known and it is related to the ability of propiconazole to inhibit the metabolization of clothianidin by cytochrome P450

monooxygenases (Berenbaum and Johnson, 2015). According to our data, a similar significant synergistic effect was also observed in the ternary mixture by considering PRO+Cr (A term) and CLO (B term) as well as CLO+Cr (A term) and PRO (B term), although within a shorter time period (4-24 h). Cr contribution to the synergistic effect observed in the ternary mixture with clothianidin and propiconazole was ruled out by considering the effect size of CLO+PRO (A term) and Cr (B term).

A significant ($p < 0.05$) antagonistic effect in the chromium and propiconazole mixture was revealed at 72 and 96 hours after ingestion, according to the negative effect size values observed. In the literature, no information to explain the observed antagonistic effect is available.

To exclude any possible complexation of propiconazole by Cr(III) able to decrease the lethal effect of these stressors in honey bees, a UV study on syrup solution containing propiconazole and Cr as single compounds and their combination were performed both at the concentration adopted in the mixture as well as at one order of magnitude higher. The UV spectra (data not shown) did not reveal visible absorption differences, thus excluding any propiconazole-Cr complex formation. Likely, the antagonism between propiconazole and Cr may affect their main physiological detoxification processes in honey bees as bioavailability, uptake, internal transportation, metabolization, binding at the target site and excretion.

5. Conclusions

The calculated LD_{50} of chromium as $Cr(NO_3)_3$ in *A. mellifera* adults (2049 mg L^{-1} syrup solution or $20.5 \text{ } \mu\text{g bee}^{-1}$) indicates low toxicity. Acute exposure to Cr at concentration higher than 379 mg L^{-1} (BMDL) may cause lethal effects to honey bee foragers. However, these concentrations are 10-100 times higher than the level usually found in honey bee matrices, thus confirming moderate Cr risks for honey bee foragers. In addition, honey bees showed higher ability to eliminate Cr (low Cr MBR)

349 compared to other heavy metals (Al, Pb, Cd and Fe). However, Cr effect on mortality of bee larvae
350 or behavioural perturbation that might chronically affect colony could not be ruled out.

351 Chromium at environmental concentration (3.9 mg L^{-1}) ingested alone or in combination with
352 sublethal doses of clothianidin and propiconazole did not significantly decrease the survival rate in
353 bees. A modified binomial proportion test-based method was developed to analyse pairwise
354 synergistic and antagonistic interactions between the three stressors for each assessment time.
355 Significant synergistic effects were observed in bees in the first 48 hours after ingestion in the
356 mixture clothianidin and propiconazole either in the presence or in the absence of chromium,
357 whereas antagonistic effects were observed in the binary mixture of propiconazole and Cr at 72 and
358 96 hours after ingestion.

359

360 **Competing interests**

361 We have no competing interests.

362

363 **Acknowledgements**

364 This study was supported by the University of Bologna (Grant
365 RFO2015_2016_BRASCHI_ILARIA). Dr Marco Montanari and Dr Andrea Simoni are
366 acknowledged for their assistance in lab trials. The reviewers' constructive and helpful comments
367 were highly appreciated.

368

369 **Reference**

370 Abbo, P.M., Kawasaki, J.K., Hamilton, M., Cook, S.C., DeGrandi-Hoffman, G., Li W.F., Liu, J.,
371 Chen Y.P., 2017. Effects of Imidacloprid and *Varroa destructor* on survival and health of
372 European honey bees, *Apis mellifera*. Insect. Sci. 24 (3), 467-477. doi: 10.1111/1744-
373 7917.12335.

374 Berenbaum, M.R., Johnson R.M., 2015. Xenobiotic detoxification pathways in honey bees. Curr.
375 Opin. Insect. Sci. 10, 51–58.

376 Biddinger, D.J., Robertson, J.L., Mullin, C., Frazier, J., Ashcraft, S., Rajotte, E.G., et al., 2013.
377 Comparative toxicities and synergism of apple orchard pesticides to *Apis mellifera* (L.) and
378 *Osmia cornifrons* (Radoszkowski). PloS One 8(9), e72587.
379 <http://doi.org/10.1371/journal.pone.0072587>.

380 Biesmeijer, J.C., Roberts, S.P.M., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., et al.,
381 2006. Parallel Declines in Pollinators and Insect-Pollinated Plants in Britain and the
382 Netherlands. Science, 313 (5785), 351–354. <http://doi.org/10.1126/science.1127863>.

383 Chaplin-Kramer, R., Dombeck, E., Gerber, J., Knuth, K.A., Mueller, N.D., Mueller, M., et al.,
384 2014. Global malnutrition overlaps with pollinator-dependent micronutrient production. Proc.
385 R. Soc. B 281: 20141799. <http://dx.doi.org/10.1098/rspb.2014.1799>.

386 Chauzat, M.-P., Jacques, A., Laurent, M., Bougeard, S., Hendriks, P., & Ribière-Chabert, M., 2016.
387 Risk indicators affecting honeybee colony survival in Europe: one year of surveillance.
388 Apidologie 47, 348-378. <http://doi.org/10.1007/s13592-016-0440-z>.

389 Cluzeau, S., 2002. Risk assessment of plant protection products on honey bees. In: Honey Bees:
390 Estimating the Environmental Impact of Chemicals. Edited by James Devillers and Minh-Hà
391 Pham-Delègue. London and New York. Taylor and Francis.

392 Conti, M.E., Botrè, F., 2001. Honeybees and their products as potential bioindicators of heavy
393 metals contamination. Environ. Monit. Assess. 69(3), 267–82.

394 Dance, C., Botías, C., Goulson, D., 2017. The combined effects of a monotonous diet and exposure
 395 to thiamethoxam on the performance of bumblebee micro-colonies. *Ecotoxicol. Environ.*
 396 *Safety* 139, 194-201. <https://doi.org/10.1016/j.ecoenv.2017.01.041>.

397 Di, N., Hladun, K.R., Zhang, K., Liu, T.X., Trumble, J.T., 2016. Laboratory bioassays on the
 398 impact of cadmium, copper and lead on the development and survival of honeybee (*Apis*
 399 *mellifera* L.) larvae and foragers. *Chemosphere* 152, 530-538.

400 Di Bona, K.R., Love, S., Rhodes, N.R., McAdory, D., Sinha, S.H., Kern, N., et al., 2011.
 401 Chromium is not an essential trace element for mammals: effects of a “low-chromium” diet. *J*
 402 *Biol. Inorg. Chem.* 16, 381–390. <http://doi.org/10.1007/s00775-010-0734-y>.

403 EFSA (European Food Safety Authority), 2009. Guidance of the Scientific Committee on a request
 404 from EFSA on the use of the benchmark dose approach in risk assessment. *EFSA J.* 7(6),
 405 1150. <http://doi.org/10.2903/j.efsa.2009.1150>.

406 EFSA (European Food Safety Authority), 2013a. Conclusion on the peer review of the pesticide
 407 risk assessment for bees for Clothianidin. *EFSA J.* 11(1), 3066.
 408 <http://doi.org/10.2903/j.efsa.2013.3066>.

409 EFSA (European Food Safety Authority), 2013b. Reasoned opinion on the review of the existing
 410 maximum residue levels (MRLs) for ethoxyquin according to Article 12 of Regulation (EC).
 411 *EFSA J.* 11(396), 3231. <http://doi.org/10.2903/j.efsa.2013.3231>.

412 EFSA (European Food Safety Authority), 2017. Update: Guidance on the use of the benchmark
 413 dose approach in risk assessment. *EFSA J.* 15(1), 4658. doi:10.2903/j.efsa.2017.4658.

414 Exley, C., Rotheray, E., Goulson, D., 2015. Bumblebee pupae contain high levels of aluminium.
 415 *PloS One* 10(6), e0127665. <http://doi.org/10.1371/journal.pone.0127665>.

416 Fauser-Misslin, A., Sadd, B. M., Neumann, P., Sandrock, C., 2014. Influence of combined pesticide

417 and parasite exposure on bumble bee colony traits in the laboratory. J. Appl. Ecol. 51, 450–
418 459. <http://doi.org/10.1111/1365-2664.12188>.

419 Finney, D.J., 1952. Probit Analysis. 2nd edition. Cambridge University Press. 318 pp.

420 Gao, Q., Wang, X.Y., Wang, W.Q., Lei, C.L., Zhu, F., 2017. Influences of chromium and cadmium
421 on the development of black soldier fly larvae. Environ. Sci. Pollut. Res. 24, 8637-8644.

422 Gauthier, M., Aras, P., Jumarie, C., Boily, M., 2016. Low dietary levels of Al, Pb and Cd may
423 affect the non-enzymatic antioxidant capacity in caged honey bees (*Apis mellifera*).
424 Chemosphere 144, 848–854. <http://doi.org/10.1016/j.chemosphere.2015.09.057>.

425 Goulson, D., Nicholls, E., Botías, C., Rotheray, E.L., 2015. Bee declines driven by combined stress
426 from parasites, pesticides, and lack of flowers. Science 347, 1255957-1-1255957-9.
427 <http://doi.org/10.1126/science.1255957>.

428 Heard, M. S., Baas, J., Dorne, J.-Lou, Lahive, E., Robinson, A.G., Rortais, A., et al., 2017.
429 Comparative toxicity of pesticides and environmental contaminants in bees: Are honey bees a
430 useful proxy for wild bee species? Sci. Total. Environ. 578, 357–365.
431 <http://doi.org/10.1016/j.scitotenv.2016.10.180>.

432 Hepburn, D.D.D, Xiao, J., Bindom, S., Vincent, J.B., O'Donnell, J., 2003. Nutritional supplement
433 chromium picolinate causes sterility and lethal mutations in *Drosophila melanogaster*. PNAS
434 100, 3766–3771.

435 Hladun, K.R., Kaftanoglu, O., Parker, D.R., Tran, K.D., Trumble, J.T., 2013. Effects of selenium on
436 development, survival, and accumulation in the honeybee (*Apis mellifera* L.). Environ.
437 Toxicol. Chem. 32, 2584–2592. <http://doi.org/10.1002/etc.2357>.

438 Holm, S., 1979. A simple sequentially rejective multiple test procedure. Scand. J. Stat. 6, 65–70.

439 Jumarie, C., Aras, P., Boily, M., 2017. Chemosphere Mixtures of herbicides and metals affect the
 440 redox system of honey bees. Chemosphere 168, 163–170.
 441 <http://doi.org/10.1016/j.chemosphere.2016.10.056>

442 Klein, A.-M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C.,
 443 Tscharntke, T., 2007. Importance of pollinators in changing landscapes for world crops. Proc.
 444 R. Soc. B 274, 303–313. <http://doi.org/10.1098/rspb.2006.3721>.

445 Ladurner, E., Bosch, J., Kemp, W.P., Maini, S., 2005. Assessing delayed and acute toxicity of five
 446 formulated fungicides to *Osmia lignaria* Say and *Apis mellifera*. Apidologie 36, 449–460.

447 Lambert, O., Piroux, M., Puyo, S., Thorin, C., L’Hostis, M., Wiest, L., et al., 2013. Widespread
 448 Occurrence of Chemical Residues in Beehive Matrices from Apiaries Located in Different
 449 Landscapes of Western France. PLoS One 8(6), 1–12.
 450 <http://doi.org/10.1371/journal.pone.0067007>.

451 Lautenbach, S., Seppelt, R., Liebscher, J., Dormann, C.F., 2012. Spatial and temporal trends of
 452 global pollination benefit. PloS One, 7(4), e35954.
 453 <http://doi.org/10.1371/journal.pone.0035954>.

454 Lee, K.V., Steinhauer, N., Rennich, K., Wilson, M.E., Tarpy, D.R., Caron, D.M., et al., 2015. A
 455 national survey of managed honey bee 2013–2014 annual colony losses in the USA.
 456 Apidologie 46, 292–305. <http://doi.org/10.1007/s13592-015-0356-z>.

457 Li, L.J., Zhang, F., Liu, X.M., Guo, Y.P., Ma, E.B., 2005. Oxidative stress related enzymes in
 458 response to chromium(VI) toxicity in *Oxya chinensis* (Orthoptera: Acridoidae). J. Environ. Sci.
 459 17, 823–826.

460 Medrzycki, P., 2013. Funnel trap – a tool for selective collection of exiting forager bees for tests. J.
 461 Apic. Res. 52, 122–123. <http://doi.org/10.3896/IBRA.1.52.3.02>.

462 Medrzycki, P., Giffard, H., Aupinel, P., Belzunces, L.P., Chauzat, M.-P., Claßen, C., et al., 2013.
 463 Standard methods for toxicology research in *Apis mellifera*. J. Apic. Res. 52, 1–60.
 464 <http://doi.org/10.3896/IBRA.1.52.4.14>.

465 Moroń, D., Grześ, I.M., Skórka, P., Szentgyörgyi, H., Laskowski, R., Potts, S.G., Woyciechowski,
 466 M., 2012. Abundance and diversity of wild bees along gradients of heavy metal pollution. J.
 467 Appl. Ecol. 49(1), 118–125. <http://doi.org/10.1111/j.1365-2664.2011.02079.x>.

468 Mullin, C.A, Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., Vanengelsdorp, D., Pettis, J.S.,
 469 2010. High levels of miticides and agrochemicals in North American apiaries: implications for
 470 honey bee health. PloS One 5(3), e9754. <http://doi.org/10.1371/journal.pone.0009754>.

471 OECD, 1998. Guideline for testing of chemicals. Test No. 213: Honey bees, acute oral toxicity test.
 472 OECD, Paris, France (1998).

473 Oliveira, H., 2012. Chromium as an environmental pollutant: Insights on induced plant toxicity.
 474 Journal of Botany, Article ID 375843, 1-8. <http://doi.org/10.1155/2012/375843>.

475 Ollerton, J., Winfree, R., Tarrant, S., 2011. How many flowering plants are pollinated by animals?
 476 Oikos 120(3), 321–326. <http://doi.org/10.1111/j.1600-0706.2010.18644.x>.

477 Pistorius J., Wehner A., Kriszan M., Barga H., Knäbe S., Klein O., Frommberger M., Stähler M.,
 478 Heimbach U., 2015. Application of predefined doses of neonicotinoid containing dusts in field
 479 trials and acute effects on honey bees. B Insectology 68, 161-172.

480 Porrini, C., Ghini, S., Girotti, S., Sabatini, A.G., Gattavecchia, E., Celli, G., 2002. Use of honey
 481 bees as bioindicators of environmental pollution in Italy. In: Honey Bees: Estimating the
 482 Environmental Impact of Chemicals. Edited by James Devillers and Minh-Hà Pham-Delègue.
 483 London and New York. Taylor and Francis.

484 Porrini, C., Mutinelli, F., Bortolotti, L., Granato, A., Laurenson, L. et al., 2016. The Status of

485 Honey Bee Health in Italy: Results from the Nationwide Bee Monitoring Network. PLoS One,
 486 11(5): e0155411. <http://doi.org/10.1371/journal.pone.0155411>.

487 Potts, S., Roberts, S., Dean, R., Marris, G., Brown, M., Jones, R., et al., 2010. Declines of managed
 488 honey bees and beekeepers in Europe. J. Apic. Res. 49, 15-22. DOI: 10.3896/IBRA.1.49.1.02.

489 Rehwoldt, R., Lasko, L., Shaw, C., Wirhowski, E., 1973. The acute toxicity of some heavy metal
 490 ions toward benthic organisms. Bull. Environ. Contam. Toxicol. 10, 291-294.

491 Robinson, A., Hesketh, H., Lahive, E., Horton, A.A., Svendsen, C., Rortais, A., et al., 2017.
 492 Comparing bee species responses to chemical mixtures: Common response patterns? PLoS
 493 One 12(6): e0176289. <https://doi.org/10.1371/journal.pone.0176289>.

494 Satta, A., Verdinelli, M., Ruiu, L., Buffa, F., Salis, S., Sassu, A., Floris, I., 2012. Combination of
 495 beehive matrices analysis and ant biodiversity to study heavy metal pollution impact in a post-
 496 mining area (Sardinia, Italy). Environ. Sci. Pollut. Res. 19(9), 3977–3988.
 497 <http://doi.org/10.1007/s11356-012-0921-1>

498 Sgolastra, F., Medrzycki, P., Bortolotti, L., Renzi, T., Tosi, S., Bogo, G., et al. 2017. Synergistic
 499 mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting
 500 fungicide in three bee species. Pest. Manag. Sci. 73, 1236–1243.
 501 <http://doi.org/10.1002/ps.4449>

502 Simon-Delso, N., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Chagnon, M., Downs, C.,
 503 Wiemers, M., 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of
 504 action and metabolites. Environ. Sci. Pollut. Res. 22, 5–34. [http://doi.org/10.1007/s11356-](http://doi.org/10.1007/s11356-014-3470-y)
 505 [014-3470-y](http://doi.org/10.1007/s11356-014-3470-y).

506 Sorensen, M.A., Jensen, P.D., Walton, W.E., Trumble, J.T., 2006. Acute and chronic activity of
 507 perchlorate and hexavalent chromium contamination on the survival and development of *Culex*

508 *quinquefasciatus* Say (Diptera: Culicidae). Environ. Pollut. 144, 759-764.

509 Svoboda, J., 1961. Prumyslové otravy vcel arsenem (Industrial poisoning of bees by arsenic). Ved.
510 Pr. Vyzk. Ustavu Vcelarskeho CSAZV 2, 55–60.

511 Thompson, H.M., Fryday, S.L., Harkin, S., Milner, S., 2014. Potential impacts of synergism in
512 honeybees (*Apis mellifera*) of exposure to neonicotinoids and sprayed fungicides in crops.
513 Apidologie 45, 545–553. <http://doi.org/10.1007/s13592-014-0273-6>.

514 Tomlin, C.D.S., 2003. ed. The e-Pesticide Manual: a World Compendium. 13th ed. Surrey, UK:
515 British Crop Protection Council. Version 3.0.

516 Tucker, F.B., Wang, K., Lu, S., Xu, L., 2003. The influence of form and quantity of chromium on
517 the development and survival of two silkworm (*Bombyx mori* L.) races. J. Environ. Sci. 15,
518 744-748.

519 Warnick S.L., Bell H.L., 1969. The Acute Toxicity of Some Heavy Metals to Different Species of
520 Aquatic Insects. J. Water Pollut. Control. Fed. 41, 280-284.

521 WSDA (Washington State Department of Agriculture), 2010. Pollinator protection requirements for
522 Section 18 Emergency Exemptions and Section 24(c) special local need registration in
523 Washington State. (AGR PUB 631–225.) 9 pp. Olympia, WA: Registration Services Program,
524 Pesticide Management Division, Washington State Department of Agriculture.

525 Wu, G., Yi, Y., 2015. Effects of dietary heavy metals on the immune and antioxidant systems of
526 *Galleria mellonella* larvae. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 167, 131-139.

527 Zayed, A.M., Terry, N., 2003. Chromium in the environment: Factors affecting biological
528 remediation. Plant and Soil 249, 139–156. <http://doi.org/10.1023/A:1022504826342>.

529 Zimmerman, D.W., Zumbo, B.D., 1993. Relative power of the Wilcoxon test, the Friedman test,

530 and repeated measures ANOVA on ranks. J. Exp. Educ. 62, 75-86.

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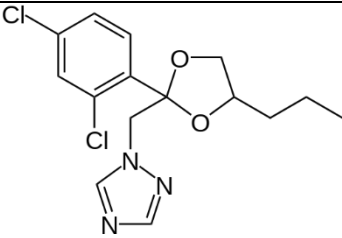
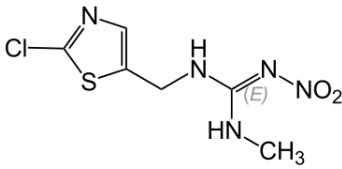
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549 **Table 1.** Main chemical characteristics of agrochemicals under investigation.

Chemical structure	Abbreviation	Molecular weight (g mol ⁻¹)	p <i>Ka</i>
	PRO	342.22	1.09*
	CLO	249.67	11

550 * p*Ka* of the conjugate acid (Tomlin, 2003)

551

552 **Table 2.** Lowest and highest benchmark doses* (BMDL and BMDU, respectively) and lethal
553 dose** (LD₅₀) of Cr following acute oral exposure to Cr(NO₃)₃ or Cr₂(SO₄)₃ in *Apis mellifera* at 48
554 h after ingestion. In brackets, the 95% CLs for LD₅₀ values.

Compound	BMDL-BMDU mg Cr L ⁻¹	LD ₅₀ (±95% CLs)			
		χ^2	p	mg Cr L ⁻¹	µg Cr bee ⁻¹
<i>Cr(NO₃)₃·9H₂O</i>	379-1670	0.341	>0.05	2049 (1674-2508)	20.5 (16.7-25.1)
<i>Cr₂(SO₄)₃</i>	43-1250	0.270	>0.05	3458 (1917-6237)	34.6 (19.2-62.4)

555 *Obtained with PROAST version 62.5; **Obtained with Probit analysis

556

557 **Table 3.** Pairwise p comparison results obtained with Holm-Sidak multicomparison test based on
558 Log-rank Kaplan-Meier survival analyses. Significantly different comparison with p <0.05 (PRO:
559 propiconazole; CLO: clothianidin; Negative control: sugar syrup solution; Solvent control: sugar
560 syrup solution with 1.5% acetone).

Pairwise p comparison	Negative control	Solvent control	Cr	CLO	PRO	CLO+PRO	PRO+Cr	CLO+Cr
Solvent control	0.925	-	-	-	-	-	-	-
Cr	0.439	0.923	-	-	-	-	-	-
CLO	0.161	0.843	0.952	-	-	-	-	-
PRO	0.91	0.857	0.954	0.899	-	-	-	-
CLO+PRO	<0.001	<0.001	<0.001	0.002	<0.001	-	-	-
PRO+Cr	0.927	941	0.906	0.67	0.947	<0.001	-	-
CLO+Cr	0.001	0.044	0.425	0.857	0.069	0.183	0.022	-
CLO+PRO+Cr	<0.001	0.002	0.035	0.18	0.004	0.942	0.001	0.923

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Table 4. Effect size for binary (PRO+CLO; PRO+Cr, CLO+Cr) and ternary (PRO+CLO+Cr) mixtures at each assessment time (4, 24, 48, 72, and 96 h). A or B terms refer to the effect size of single pollutants in binary or in ternary mixture. A positive or negative difference indicates synergistic or antagonistic effect. Significance levels (Holm-corrected for multiple comparisons) for differences are shown within parentheses, i.e. (*): $p < 0.05$; (**): $p < 0.01$; (***): $p < 0.001$.

A	B	4 h	24 h	48 h	72 h	96 h
CLO	PRO	0.1900(**)	0.3650(***)	0.3181(**)	0.1322	0.0978
Cr	PRO	0.0167	0.0003	-0.1069	-0.2811(*)	-0.3244(*)
CLO	Cr	0.0342	0.0850	-0.0247	-0.1197	-0.0978
CLO+PRO	Cr	0.0973	-0.0553	-0.1467	-0.2193	-0.2307
PRO+Cr	CLO	0.2683(***)	0.3033(***)	0.2181(*)	0.0356	-0.0022
CLO+Cr	PRO	0.2500(**)	0.2200 (*)	0.1569	-0.0500	-0.1133

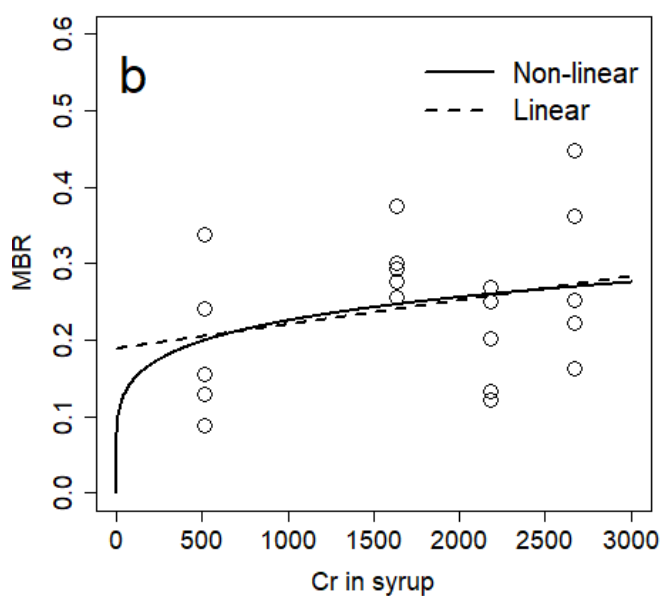
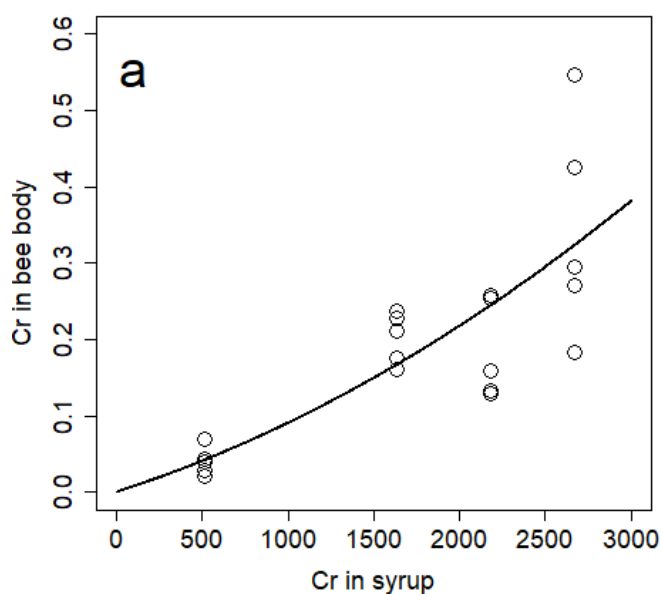
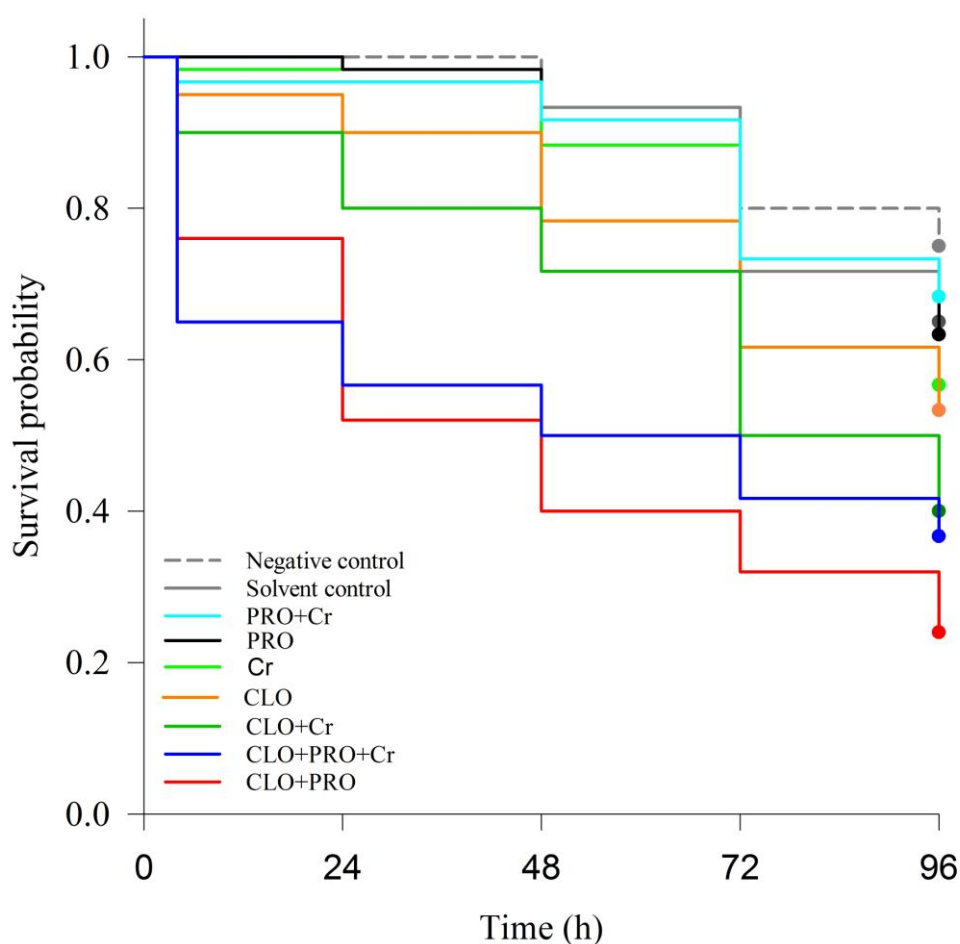


Figure 1. Results of regression analysis to the a) Cr-retained and b) MBR observations. Observational data points are shown as empty dots. Figures also show a) parabola (solid line) and b) nonlinear (solid line) and linear (dashed line) curves fitted to the data. Analytic expressions for each

584 curve can be found in the Supplementary data. The parabola in a) and the non-linear curve in b) are
 585 forced to pass through the origin of coordinates (0, 0).

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589 Figure 2. Cumulative proportion of surviving *Apis mellifera* foragers orally exposed to
 590 propiconazole (PRO, 700 mg L⁻¹), clothianidin (CLO, 0.074 mg L⁻¹) and Cr (3.9 mg L⁻¹) as single
 591 pollutants or binary and ternary mixtures. Negative control (sugar syrup solution) and solvent
 592 control (sugar syrup solution with 1.5% acetone) are reported for comparison.

593