

Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops

Article (Submitted Version)

David, Arthur, Botias, Cristina, Abdul-Sada, Alaa, Nicholls, Elizabeth, Rotheray, Ellen L, Hill, Elizabeth M and Goulson, Dave (2016) Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. *Environment International*, 88. pp. 169-178. ISSN 0160-4120

This version is available from Sussex Research Online: <http://sro.sussex.ac.uk/id/eprint/59217/>

This document is made available in accordance with publisher policies and may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher's version. Please see the URL above for details on accessing the published version.

Copyright and reuse:

Sussex Research Online is a digital repository of the research output of the University.

Copyright and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners. To the extent reasonable and practicable, the material made available in SRO has been checked for eligibility before being made available.

Copies of full text items generally can be reproduced, displayed or performed and given to third parties in any format or medium for personal research or study, educational, or not-for-profit purposes without prior permission or charge, provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.

1 **Widespread contamination of wildflower and bee-collected pollen**
2 **with complex mixtures of neonicotinoids and fungicides.**

3

4

5 Arthur David*, Cristina Botías, Alaa Abdul-Sada, Elizabeth Nicholls, Ellen L. Rotheray, Elizabeth
6 M. Hill and Dave Goulson

7 School of Life Sciences, University of Sussex, Brighton, U.K. BN1 9QG

8

9

10 *To whom correspondence should be addressed:

11 Tel: +44 1273 672961,

12 Fax: 44 1273 877586,

13 email: arthur.david@sussex.ac.uk

14

15 **Abstract**

16 There is considerable and ongoing debate as to the harm inflicted on bees by exposure to
17 agricultural pesticides. In part, the lack of consensus reflects a shortage of information on field-
18 realistic levels of exposure. Here, we quantify concentrations of neonicotinoid insecticides and
19 fungicides in the pollen of oilseed rape, and in pollen of wildflowers growing near arable fields. We
20 then compare this to concentrations of these pesticides found in pollen collected by honey bees and
21 in pollen and adult bees sampled from bumblebee colonies placed on arable farms. We also
22 compared this with levels found in bumblebee colonies placed in urban areas. Pollen of oilseed rape
23 was heavily contaminated with a broad range of pesticides, as was the pollen of wildflowers growing
24 nearby. Consequently, pollen collected by both bee species also contained a wide range of
25 pesticides, notably including the fungicides carbendazim, boscalid, flusilazole, metconazole,
26 tebuconazole and trifloxystrobin and the neonicotinoids thiamethoxam, thiacloprid and
27 imidacloprid. In bumblebees, fungicides carbendazim, boscalid, tebuconazole, flusilazole and
28 metconazole were present at concentrations up to 73 nanogram/gram (ng/g). Pesticide
29 concentrations in pollen collected by honeybees tended to be lower than those in pollen collected
30 by bumblebees. It is notable that pollen collected by bumblebees in rural areas contained high levels
31 of the neonicotinoids thiamethoxam (mean 18 ng/g) and thiacloprid (mean 2.9 ng/g), along with a
32 range of fungicides, some of which are known to act synergistically with neonicotinoids. Pesticide
33 exposure of bumblebee colonies in urban areas was much lower than in rural areas. Understanding
34 the effects of simultaneous exposure of bees to complex mixtures of pesticides remains a major
35 challenge.

36 **Keywords:** neonicotinoids, fungicides, pollen, bumblebees, honeybees

37 Introduction

38 The extent, causes and consequences of bee declines have attracted much scientific and public
39 attention in the last decade. It is clear that there is no single cause, but that several interacting
40 factors including declines in floral abundance and diversity resulting from agricultural intensification,
41 the spread of parasites and pathogens, and exposure to pesticides all contribute to these declines
42 (Goulson et al., 2015). The impact of pesticides, in particular the class of insecticides known as
43 neonicotinoids, on pollinator declines is the most controversial of these factors.

44 Neonicotinoids are neurotoxins which act as nicotinic acetylcholine receptor agonists in the central
45 nervous system of insects and cause overstimulation, paralysis, and death (Goulson 2013). These
46 pesticides are systemic and are widely applied as seed dressings to flowering crops, where they can
47 be detected at the low ng/g level in the nectar and pollen (Fairbrother et al., 2014). Pollen is a major
48 food source for growing larvae and nurse workers, and so is a likely source of exposure of bees to
49 neonicotinoids (Sanchez-Bayo and Goka 2014).

50 A key part of the debate over the impacts of neonicotinoids has become focussed on the dose that
51 bees are likely to be exposed to in the field. Laboratory and semi-field studies are often dismissed as
52 using unrealistically high doses of pesticides. For example Whitehorn et al. (2012) experimentally
53 exposed bumblebee colonies to pollen containing 6 ng/g of the neonicotinoid imidacloprid, plus 0.70
54 ng/g in their nectar, and found an 85% drop in queen production compared to controls. However, it
55 has since been argued that this dose was higher than bumblebees are likely to receive in the field
56 because colonies will be feeding on a mix of contaminated crops and uncontaminated wildflowers
57 (Carreck and Ratnieksi 2014). Thus obtaining more information on what constitutes field realistic
58 exposure to both bumblebee and honey bee colonies is vital to taking this debate forwards.

59 In addition to neonicotinoids, there is clear evidence that honey bees are routinely exposed to a
60 complex mixture of many different agrochemicals (Johnson et al., 2012). An analysis of honey bees
61 and their hive wax and pollen in the USA revealed that the majority of samples were contaminated
62 with at least one pesticide, and a total of 121 different agrochemicals, including metabolites and
63 miticides, were detected in samples (Mullin et al., 2010). Similarly 37 insecticide and fungicide
64 chemicals were detected in honey bees and hive products sampled in France (Lambert et al., 2013).
65 In addition to the active ingredients, bees may also be exposed to additives used in pesticide
66 formulations and these have also been detected in pollen and honey with the potential to interact
67 with pesticides and increase toxic effects (Mullin et al., 2015). Synergistic toxicity of some
68 combinations of insecticides and fungicides have been reported for honey bees or their larvae (Iwasa

69 et al., 2004; Schmuck et al., 2003; Thompson et al., 2014; Zhu et al., 2014). For example the toxicity
70 of some neonicotinoids can be increased by as much as a factor of 1000 by simultaneous exposure
71 to demethylation inhibiting (DMI) fungicides (Iwasa et al., 2004; Schmuck et al., 2003). DMI
72 fungicides act by inhibiting Cytochrome P450 (CYP P450) mediated ergosterol biosynthesis in fungi
73 and are thought to inhibit CYP P450 enzymes in insects that are important for detoxification of
74 neonicotinoids and other insecticides (Schmuck et al., 2003).

75 Our study focusses on determining which mixtures of commonly used fungicides occur alongside
76 neonicotinoids in crop and wildflower pollen and in the pollen collected by honey bees and
77 bumblebees. Our aim is to investigate the potential for exposure of bees to mixtures of
78 neonicotinoid and fungicide pesticides which are present in crop and wildflower pollen. Pesticides
79 were analysed in pollen collected from oilseed rape (OSR) flowers, wildflowers growing in margins of
80 OSR and winter wheat (WW) crops, and from pollen collected by honey bee (*Apis mellifera*) and
81 bumblebee (*Bombus terrestris*) colonies placed in arable farmland. We also compare exposure of
82 bumblebee nests placed in urban versus rural areas, and quantify residues in the adult bumblebees.
83 Mixtures of a total of 20 agrochemicals were analysed comprising neonicotinoids and fungicides
84 commonly used in UK crops.

85

86 **2. Material and methods**

87 2.1 Sample collection

88 2.1.1 Pollen collected from plants

89 - *OSR pollen*

90 Pollen samples from OSR flowers were collected in 7 fields from three farms located in East Sussex
91 (United Kingdom) during the OSR blooming period (end of May – June 2013), and from 1 to 3 sites per
92 OSR field were sampled (n=11 in total). To obtain pollen samples, OSR flowers were gathered, stored
93 on ice in coolers in the field and then frozen immediately at -80°C until further handling. At processing,
94 flower samples were gently defrosted and dried in an incubator at 37 °C for 24 hours to facilitate
95 pollen release from the anthers. After drying, flowers were brushed over food strainers to separate
96 pollen from anthers and sifted through multiple sieves of decreasing pore sizes (pore sizes from 250
97 to 45 µm).

98 - *Wild plants in the field margins.*

99 Wildflowers pollen samples were collected from 4 of the 7 OSR fields as well as in the margin of 4 WW
100 fields present in same the 3 farms. Field boundaries in the region typically consist of a hedge of woody
101 plants separated from the crop by a 0-2 m strip of herbaceous vegetation. The average sample
102 distance from the crop edge was 1.5 m (range 1-2 m). Samples of pollen were collected from the
103 wildflowers present in the field margins and hedge using the method described above for OSR plants.
104 The species of wildflowers collected depended upon which species were available. In OSR field
105 margins, pollen from 8 wildflower samples comprising 4 different species (*Ranunculus repens*, *Silene*
106 *latifolia* (sampled 3 times), *Matricaria recutita* (x3), *Cirsium vulgare*) were collected. In WW margins,
107 pollen from 13 wildflower samples comprising 8 different species (*Heracleum sphondylium* (x4),
108 *Papaver rhoeas*, *Cirsium vulgare*, *Senecio jacobaea*, *Rosa canina*, *Pimpinella saxifraga*, *Aethusa*
109 *cynapium* and *Matricaria recutita* (x3)) were collected. Pollen samples were analysed separately from
110 each species with the exception of low amounts (< 20 mg) of four wildflower pollen samples collected
111 from plants growing at the same site of a WW margin which were pooled and analysed as a single
112 sample.

113 2.1.2 Pollen collected from bees.

114 - honey bees

115 Five honey bee (*Apis mellifera*) colonies were placed in the vicinity of the OSR fields at the beginning
116 of the OSR flowering period (May 2013) and stayed in the same sites until the end of August 2013.
117 Distances between the hives and the nearest OSR fields ranged from 1 to 260 m (see Table S1). The
118 hives were equipped with pollen traps during 4 consecutive days at the beginning of June 2013 (i.e.,
119 during the OSR blooming period), and for 4 days in mid-August 2013 (i.e., when no OSR was in flower)
120 in order to collect pollen loads from the returning honey bee foragers. After 4 days, the traps were
121 removed from the hives and the pollen gathered and stored on ice in coolers in the field, and then at
122 -80 °C until analysis. Pollen balls within each sample were sorted and weighed by colour (Human et
123 al., 2013; Kirk 2006). Pollen grains associated with plant species were identified under a microscope
124 following standard methods and using reference specimens and published reference collections
125 (Demske et al., 2013; Moore et al., 1991; Sawyer 1981).

126 - bumblebees

127 Eight bumblebee nests (*Bombus terrestris audax*) were obtained from Agralan Ltd, Swindon, UK
128 (originating from Biobest, Belgium). A sample of bumblebee workers from Biobest nests was analysed
129 for target pesticides prior to the experiment and levels of all test analytes in bumblebee extracts were
130 below the method detection limits. Five nests were placed in different farmland sites in South-East

131 England (East and West Sussex) at the beginning of May 2013. Sites were at least 1 km apart and in
132 average 590 m far from the nearest OSR crop (range 8-1116 m, see Table S1). Three other nests were
133 located in gardens from urban areas of West Sussex, being separated more than 4 km apart, and with
134 an average distance to the nearest OSR crop of 1577 m (range 240-2670 m). After 4 weeks of free
135 foraging in the field (comprising most of the OSR blooming period), pollen samples (> 200 mg) were
136 collected from the in-nest stores in every colony using stainless steel micro-spoons, and were stored
137 in 1.5 ml micro-centrifuge tubes at -80° C. Before extractions, every pollen sample was manually
138 homogenised using a micro-spatula. A subsample of approximately 2 mg was evenly spread in a
139 microscope slide, using glycerine jelly as the mounting medium. Light microscopy was used to identify
140 the source of the pollen grains within the samples, and the proportion of the different taxa present in
141 the samples was estimated by identifying pollen grains in five microscope fields of view uniformly
142 distributed across the slide coverslip until 200 pollen grains were counted. After ten weeks of free
143 foraging in the field, three to eight workers per nest were also collected for pesticide analysis of
144 individual bees.

145 2.2 Pesticide analysis

146 2.2.1 Chemicals and reagents

147 Choice of analytes: Details of test analytes used in the study are given in Table 1. The pesticides
148 comprised nine classes of contaminants and included all five of the neonicotinoid chemicals that are
149 registered for use in the UK. Fungicides were chosen based on the most used (by weight) in UK crops
150 including oilseed rape, wheat, spring barley, field bean, strawberry and raspberry crops
151 (<https://secure.fera.defra.gov.uk/pusstats/surveys/2012surveys.cfm>). In addition, levels of an
152 insecticide synergist piperonyl butoxide were also analysed as it is used in agrochemical formulations
153 and has been reported to synergise the activity of some neonicotinoids (Bingham et al., 2008; Khan et
154 al., 2015).

155

156

157

158

159

160
161

Table 1. The list of chemicals analysed in this work, their chemical classes and their last applications in the studied oilseed rape (OSR) or winter wheat (WW) fields.

Chemicals	Class	Last application				Application method	Comments
		OSR field		WW field			
		Month	Year	Month	Year		
Insecticides							
Thiamethoxam	Neonicotinoid	Aug	2012	Aug	2011	seed dressing	
Clothianidin	Neonicotinoid	March	2012	Oct	2012	seed dressing	
Imidacloprid	Neonicotinoid	Not used					used prior to 2011
Acetamiprid	Neonicotinoid	Not used					used for gardening
Thiacloprid	Neonicotinoid	Not used					used in neighbouring fields in 2011 and 2012 and in gardens
Fungicides							
Carbendazim	Methyl benzimidazole carbamates (MBC)	May	2013	April	2012	spray	
Carboxin	Succinate dehydrogenase inhibitors (SDI)	Not used					commonly used for barley crops ^a
Boscalid	Succinate dehydrogenase inhibitors	May	2013	May	2013	spray	
Spiroxamine	Amines ("Morpholines") (SBI: Class II)	April	2012	June	2013	spray	
Silthiofam	Thiophene	Not used					commonly used for WW ^a
Triticonazole	Demethylation inhibitors (DMI) (SBI: Class I)*			March	2011	spray	used for gardening
Epoxiconazole	Demethylation inhibitors (SBI: Class I)	April	2012	May	2013	spray	
Tebuconazole	Demethylation inhibitors (SBI: Class I)	June	2012	June	2013	spray	used for gardening
Flusilazole	Demethylation inhibitors (SBI: Class I)	Jan	2013	Nov	2011	spray	
Prochloraz	Demethylation inhibitors (SBI: Class I)			March	2011	spray	
Metconazole	Demethylation inhibitors (SBI: Class I)	May	2013	Jan	2012	spray	
Pyraclostrobin	Quinone outside inhibitors (QoI)	April	2012	May	2013	spray	
Fluoxastrobin	Quinone outside inhibitors	May	2011	May	2011	spray	
Trifloxystrobin	Quinone outside inhibitors			May	2011	spray	used for gardening
Synergist							
Piperonyl butoxide							used in the formulation of insecticides

162 ^a information from Defra report <https://secure.fera.defra.gov.uk/pusstats/surveys/2012surveys.cfm>.

163 * SBI = sterol biosynthesis inhibitor also known as Ergosterol biosynthesis inhibitor (EBI) - an inhibitor of sterol synthesis, which is essential for fungal growth. EBI
164 fungicides include DMIs as well as the morpholines and piperidines.

165

166 Certified standards of carbendazim, thiamethoxam, thiamethoxam-d3, clothianidin, clothianidin-d3,
167 imidacloprid, imidacloprid-d4, acetamiprid, thiacloprid, carboxin, boscalid, spiromaxamine, silthiofam,
168 triticonazole, epoxiconazole, tebuconazole, flusilazole, prochloraz, metconazole, pyraclostrobin,
169 trifloxystrobin, fluoxastrobin, piperonyl butoxide and also formic acid, ammonium formate,
170 magnesium sulphate, sodium acetate and Supel™ QuE PSA/C18/GCB (ratio 1/1/1) were obtained from
171 Sigma Aldrich UK. Certified standards of carbendazim-d3 and tebuconazole-d6 were purchased from
172 LGC standards UK and prochloraz-d7 and carbamazepine-d10 from QMX Laboratories Limited UK. All
173 pesticide standards were > 99% compound purity (except triticonazole: 98.8%, spiromaxamine: 98.5%
174 and piperonyl butoxide: 97.9%) and deuterated standards > 97% isotopic purity. HPLC grade
175 acetonitrile, toluene, methanol and water were obtained from Rathburns UK. Individual standard
176 pesticide (native and deuterated) stock solutions (1 mg/ml) were prepared in acetonitrile (ACN) as
177 was an internal standard mixture of the seven deuterated pesticides at 100 ng/ml. Calibration points
178 in H₂O:ACN (70:30) were prepared weekly from the stock solutions. All solutions were stored at -20°C
179 in the dark.

180 2.2.2 Sample preparation for neonicotinoid analyses

181 - *Pollen samples*

182 Pollen samples were extracted as described in David et al. (2015). Briefly, 100 mg (\pm 5 mg) of pollen
183 sample was weighed and 400 μ g of the mix of deuterated internal standards in ACN were added to
184 each sample which was then extracted using a modified QuEChERS method. First, 400 μ l of water was
185 added and samples were then extracted by adding 500 μ l of ACN and mixing on a multi axis rotator
186 for 10 min. Then, 250 mg of magnesium sulphate: sodium acetate mix (4:1) was added to each tube.
187 After centrifugation (13,000 RCF for 5 min), the supernatant was removed into a clean Eppendorf tube
188 containing 50 mg of Supel™ QuE PSA/C18/GCB and vortexed (10 s). The extract was mixed on a multi-
189 axis rotator (10 min) and then centrifuged (10 min). The supernatant was transferred into a glass tube.
190 The PSA/C18/GCB phase was then extracted with ACN/toluene (3/1, 150 μ l vortex 15 s). After
191 centrifugation, the supernatant was combined with that of the previous ACN extract and spin filtered
192 (0.22 μ m). The extract was evaporated to dryness under vacuum, and finally reconstituted with 120
193 μ l ACN:H₂O (30:70). Finally, the extract was centrifuged for 20 min and the supernatant stored at -
194 20°C in the dark until analysis.

195 - *Bumblebee samples*

196 Bumblebees were first checked for adhering pollen residues in order to remove them before analysis.
197 Individual whole bumblebee samples were ground in liquid nitrogen with a pestle and mortar followed

198 by manual homogenisation using a micro-spatula. Each bumblebee sample was then accurately
199 weighed (average weight \pm standard deviation was 123 ± 83 mg). Then, 400 μ l of water was added
200 and the samples were homogenised for 20 s using a vortex. Samples were then extracted using the
201 same modified QuEChERS method as above (i.e, 500 μ l of ACN, 250 of magnesium sulphate: sodium
202 acetate mix (4:1) and 50 mg of PSA/C18/GCB). Extracts were reconstituted, centrifuged and stored as
203 above.

204 2.2.3 UHPLC-MS/MS analyses

205 The UHPLC-MS/MS method described in David et al. (2015) was used for the analysis of samples.
206 Briefly, sample extracts were analysed using a Waters Acquity UHPLC system coupled to a Quattro
207 Premier triple quadrupole mass spectrometer from Micromass (Waters, Manchester, UK). Pesticides
208 in extracts were separated using a reverse phase Acquity UHPLC BEH C18 column (1.7 μ m, 2.1 mm \times
209 100 mm, Waters, Manchester, UK) fitted with a ACQUITY UHPLC BEH C18 VanGuard pre-column
210 (130 \AA , 1.7 μ m, 2.1 mm \times 5 mm, Waters, Manchester, UK) and maintained at 22 $^{\circ}$ C. Injection volume
211 was 20 μ l and mobile phase solvents were 95% water, 5% ACN, 5 mM ammonium formate, 0.1% formic
212 acid (A) and 95% ACN, 5% water, 5 mM ammonium formate, 0.1% formic acid (B). Methods were
213 developed to separate all 20 test analytes within a 25 min run. The initial ratio (A:B) was 90:10 and
214 separation was achieved at 22 $^{\circ}$ C using a flow rate of 0.15 ml/min with the following gradient: 90:10 to
215 70:30 in 10 min; from 70:30 to 45:55 at 11 min, from 45:55 to 43:57 at 20 min, from 43:57 to 0:100 at
216 22 min and held for 8 min prior to return to initial conditions and equilibration for 5 min.

217 MS/MS was performed in the multiple reaction monitoring (MRM) using ESI in the positive mode and
218 two characteristic fragmentations of the deprotonated molecular ion $[M+H]^+$ were monitored for
219 quantification and confirmation (David et al., 2015). Argon was used as collision gas (P collision cell:
220 3×10^{-3} mbar), and nitrogen was used as desolvation gas (600 L/h). Mass calibration of the
221 spectrometer was performed with sodium iodide. Data were acquired using MassLynx 4.1 and the
222 quantification was carried out by calculating the response factor of neonicotinoid and fungicide
223 compounds to their respective internal standards. Analyte concentrations were determined using a
224 least-square linear regression analysis of the peak area ratio versus the concentration ratio (native
225 analyte to deuterated IS). A minimum of six point calibration curves ($R^2 > 0.99$) were used to cover the
226 range of concentrations observed in the different matrices for all compounds, within the linear range
227 of the instrument. Method detection limits (MDL) and method quantification limits (MQL) for pollen
228 and bumblebee matrices are given in Table S2.

229 2.2.4 Quality control

230 One workup sample (i.e., using extraction methods without a pollen/bee sample) per batch was
231 injected on the UHPLC-MS/MS at the beginning of the run to ensure that no contamination occurred
232 during the sample preparation. Solvent samples (ACN:H₂O (30:70)) were also injected between sample
233 batches to ensure that there was no carryover in the UHPLC system that might affect adjacent results
234 in analytical runs. Identities of detected neonicotinoids and fungicides were confirmed by comparing
235 ratios of MRM transitions in samples and pure standards. The standard calibration mixture was
236 injected before and after all sample batches to monitor sensitivity changes, and quality control
237 samples (QCs, i.e., standard solutions) were injected every 10 samples to monitor the sensitivity
238 changes during the analysis of each batch.

239 2.3 Statistical analysis

240 All statistical analyses were carried out using GraphPad Prism 6 software. Pesticide concentrations in
241 the different pollen matrices were tested for normality using the D'Agostino-Pearson test. As pesticide
242 concentrations were not normally distributed for many pesticides in the different pollen types, non-
243 parametric Mann-Whitney U-tests were used to compare the concentrations of neonicotinoids and
244 fungicides present in pollen collected from OSR flowers, wildflowers and honey bees, and for
245 bumblebees and their pollen collected from urban and rural areas. To perform the statistical analyses,
246 all concentrations that were over the limits of detection (\geq MDL) but below the limits of quantification
247 ($<$ MQL) were assigned the value considered as the MDL in each case. Concentrations below the MDL
248 were considered to be zero.

249 **3. Results**

250 3.1 Neonicotinoid and fungicide residues in pollen samples from oilseed rape, wildflowers from field
251 margins and pollen collected by honey bees.

252 3.1.1 Frequencies, ranges and mean concentrations

253 Mixtures of neonicotinoids and fungicides were analysed in pollen samples from OSR flowers,
254 wildflowers from OSR and WW margins and pollen collected by honey bees (during and after the OSR
255 bloom) in order to estimate exposure of bees to these pesticides. All the different types of pollen were
256 collected in each of the 3 different farms. Frequencies of each pesticide (i.e., percentage of samples
257 with detectable levels of pesticides) as well as the ranges, mean and median concentrations found in
258 the different pollens are presented in Table 2 (for raw data see Table S3 to S7).

259
260

Table 2. The mean, median and range of concentrations and frequency of detection of neonicotinoid and fungicide chemicals in pollen collected from oilseed rape flowers, wild flowers and by honey bees during and after the OSR bloom.

	OSR pollen				Wildflower pollen								Honeybee pollen							
	n = 11				OSR Margins n = 8				WW Margins n = 10				During OSR bloom n = 25				After OSR bloom n = 19			
	Freq %	Range ppb	Mean ppb	Median ppb	Freq %	Range ppb	Mean ppb	Median ppb	Freq %	Range ppb	Mean ppb	Median ppb	Freq %	Range ppb	Mean ppb	Median ppb	Freq %	Range ppb	Mean ppb	Median ppb
Thiamethoxam	100	2.4 - 11	5.7	3.9	50	<0.12 - 21	2.8	<0.36	30	<0.12 - 0.50	0.13	<0.12	60	<0.12 - 1.6	0.15	<0.36	21	<0.12 - <0.36		
Clothianidin	73	<0.72 - 11	3.6	3.8	0	<0.72			10	<0.72 - 5.0	0.50	<0.72	8	<0.72 - <2.2			0	<0.72		
Imidacloprid	0	<0.36			13	<0.36 - <1.1			0	<0.36			12	<0.36 - 3.5	0.20	<0.36	5	<0.36 - <1.1		
Acetamiprid	0	<0.02			0	<0.02			0	<0.02			4	<0.02 - <0.07			0	<0.02		
Thiacloprid	100	<0.22 - 78	19	7.5	63	<0.07 - 4.0	0.60	<0.22	20	<0.07 - 2.9	0.30	<0.07	48	<0.07 - 10	0.90	<0.07	0	<0.07		
Carbendazim	100	0.60 - 163	39	13	100	1.3 - 6.8	3.5	3.5	0	<0.08			96	<0.08 - 120	12	2.5	74	<0.08 - 1.4	0.40	0.34
Carboxin	0	<0.12			0	<0.12			0	<0.12			0	<0.12			0	<0.12		
Boscalid	18	<0.12 - 25	3.2	<0.12	63	<0.12 - 38	5.8	0.53	60	<0.12 - 38	8.5	1.7	52	<0.12 - 21	5.2	<0.36	37	<0.12 - 17	2.5	<0.12
Spiroxamine	100	13 - 328	80	58	88	<0.02 - 151	47	7.3	70	<0.02 - 26	7.7	6.3	28	<0.02 - 74	3.4	<0.02	47	<0.02 - 1.1	0.20	<0.02
Silthiofam	0	<0.24			0	<0.24			0	<0.24			0	<0.24			0	<0.24		
Triticonazole	0	<0.24			0	<0.24			0	<0.24			0	<0.24			0	<0.24		
Epoxiconazole	64	<0.84 - 27	4.3	2.5	0	<0.84			0	<0.84			0	<0.84			5	<0.84 - 8.3	0.40	<0.84
Tebuconazole	100	1.5 - 21	5.2	2.9	75	<0.24 - 8.5	3.3	3.2	90	<0.24 - 34	7.0	3.2	76	<0.24 - 19	1.4	<0.72	79	<0.24 - 6.4	1.2	0.85
Flusilazole	18	<0.24 - 16	1.6	<0.24	25	<0.24 - 5.0	0.80	<0.24	0	<0.24			12	<0.24 - 6.1	0.30	<0.24	0	<0.24		
Prochloraz	0	<0.36			0	<0.36			0	<0.36			0	<0.36			0	<0.36		
Metconazole	27	<0.30 - 19	2.5	<0.30	0	<0.30			0	<0.30			12	<0.30 - 12	1.0	<0.30	0	<0.30		
Pyraclostrobin	9	<0.24 - 5.4	0.50	<0.24	38	<0.24 - 4.3	1.0	<0.24	10	<0.24 - 2.8	0.30	<0.24	28	<0.24 - 9.8	0.90	<0.24	16	<0.24 - 3.7	0.40	<0.24
Trifloxystrobin	45	<0.24 - 18	2.6	<0.24	63	<0.24 - 104	13	<0.72	20	<0.24 - 1.0	0.10	<0.24	40	<0.24 - 10	1.4	<0.24	16	<0.24 - 1.0	0.10	<0.24
Fluoxastrobin	18	<0.01 - <0.02			50	<0.01 - <0.02			30	<0.01 - <0.02			12	<0.01 - <0.02			11	<0.01 - 3.9	0.20	<0.01
Piperonyl butoxide	0	<0.72			0	<0.72			0	<0.72			0	<0.72			0	<0.72		

261
262
263
264

Pollen traps were used to collect pollen brought back to honeybee hives (5) both during the OSR blooming period and later in the summer. Pollen was separated into wildflower species and analysed separately (n=3, 4, 5, 5 and 8 for hives 1, 2, 3, 4 and 5, respectively during the OSR bloom and n=5, 4, 2, 5 and 3 for hives 1, 2, 3, 4 and 5, respectively after the OSR bloom). ppb = ng/g wet weight of sample.

265

266 - *OSR flowers*

267 As expected, the number of detected pesticides, their frequencies, their ranges as well as their mean
268 concentrations were generally higher in pollen from OSR flowers than in wildflower pollen and pollen
269 collected by honey bees (Table 2). All individual OSR pollen samples contained at least 6 neonicotinoid
270 and fungicide residues and most samples contained between 7 and 12 different pesticides.
271 Thiamethoxam, thiacloprid, carbendazim and spiroxamine were the most frequently detected
272 compounds (all present in 100% of samples), followed by tebuconazole (80%) clothianidin (73%),
273 epoxiconazole (64%) and trifloxystrobin (45%). The other fungicides (i.e., boscalid, flusilazole,
274 metconazole, pyraclostrobin and fluoxastrobin) were detected in less than 30% in these samples from
275 OSR flowers. Pesticides such as carbendazim and spiroxamine were present in some samples at
276 concentrations > 100 ng/g. The range of concentrations for other fungicides were between < MDL –
277 27 ng/g, and neonicotinoid concentrations were detected at between < MDL – 78 ng/g. With the
278 exception of thiacloprid which was only applied to neighbouring fields, thiamethoxam, clothianidin,
279 carbendazim, boscalid, spiroxamine, epoxiconazole, tebuconazole flusilazole, metconazole,
280 pyraclostrobin and fluoxastrobin had been applied in the studied OSR fields in the year of the sampling
281 or up to two years before the sampling (i.e., before the rotation to OSR crop). Trifloxystrobin had been
282 applied to WW fields present in the same farms two years before the sampling period (Table 1).

283 - *Wildflower pollen*

284 Pollen from four wildflower species was collected from 8 OSR field margins between June and August
285 2013. A similar mixture of pesticides as OSR pollen was detected in pollen from wildflowers growing
286 in the OSR field margins; however their frequencies of detection and concentration ranges were
287 generally lower than for OSR pollen (Table 2, Figure 1). Concentrations of thiamethoxam (Mann-
288 Whitney test, U=11, p=0.0045) and thiacloprid (Mann-Whitney test, U=6, p=0.0006) were significantly
289 lower in wildflower pollen compared with OSR pollen. Nevertheless, it is worth nothing that the
290 highest concentration of thiamethoxam were measured in the pollen from a wildflower (21 ng/g
291 detected in pollen from *Matricaria recutita* flowers growing in the margin from OSR field 2 in farm 2,
292 Table S4). Pollen was collected from 13 wildflower samples comprising 8 different species growing in
293 8 margins of WW fields between July and August. Three neonicotinoids and six fungicides were also
294 detected in wildflower pollen collected in WW field margins, and all the agrochemicals had been
295 applied previously to WW or to nearby fields. Concentrations of most pesticides were the same in
296 pollen samples collected from the wildflowers growing in WW and OSR field margins with the
297 exception of thiacloprid (Mann-Whitney test, U=3, p=0.002) which was lower in wildflower pollen
298 from WW field margins.

300 Pollen traps were used to collect pollen brought back to honey bee hives placed on the farms, both
301 during the OSR blooming period (beginning of June 2013), and later in the summer when no OSR was
302 in flower (mid-August 2013). Honeybee pollen balls were sorted by species in order to study the
303 variability in exposure levels and sub-samples that were > 100 mg were analysed separately. (the
304 pesticide concentrations for the composite samples brought to the hives were also calculated for later
305 comparison with pollen samples collected from the bumblebee nests). During June 2013, the honey
306 bee collected pollen included 9 wildflower species and OSR pollen, and 12 wildflower species in
307 August, and the total pollen analysed comprised >86% of the total honey bee collected pollen in June
308 and >75% of the total honey bee collected pollen in August (Tables S6 and S7). In terms of weight, the
309 majority of these pollen samples collected by honey bees during the OSR flowering was from
310 wildflowers, with just 10% of pollen coming from OSR (Botías et al., 2015). All pollen samples collected
311 by honey bees were contaminated with a mixture of neonicotinoids and fungicides; a total of 14
312 compounds in pollen collected during OSR blooming and 10 after the bloom period. The number of
313 pesticides found in any one pollen sample ranged between 2 to 8 compounds. A similar mixture of
314 neonicotinoids and fungicides were detected in honey bee collected pollen in June as that present in
315 wildflowers and OSR pollen, however, these compounds were at lower concentrations in honey bee
316 corbicular pollen (Figure 1). The total concentrations of pesticides in honey bee pollen were lower in
317 August compared with June and significantly reduced for carbendazim (Mann-Whitney test, U=54,
318 $p < 0.0001$) and thiamethoxam (Mann-Whitney test, U=131.5, $p = 0.0047$). In addition, clothianidin,
319 thiacloprid, flusilazole and metconazole were no longer detected in honey bee collected pollen at this
320 time.

321

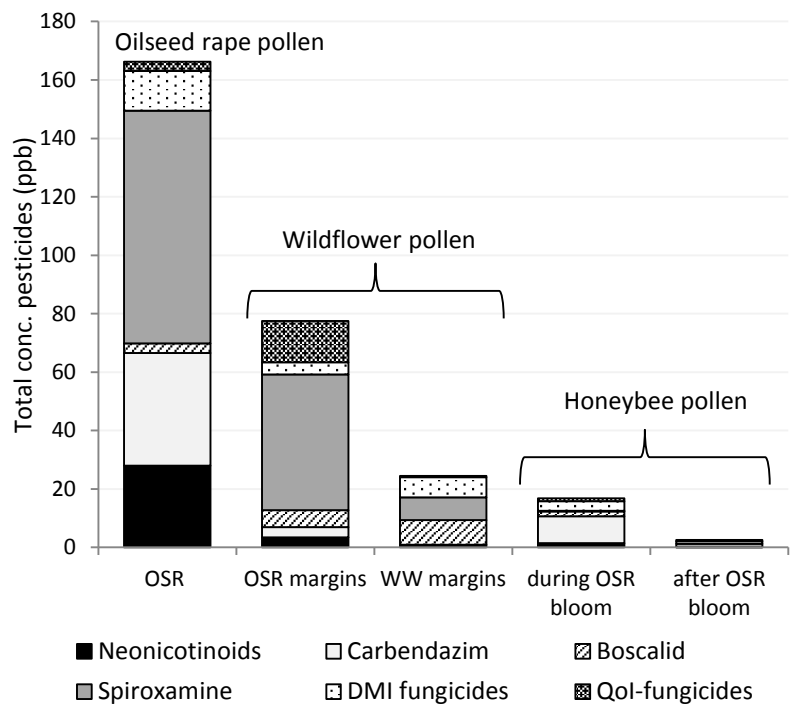
322

323

324

325

326



327

328 **Figure 1. The sum of the mean concentrations of neonicotinoids and fungicides in pollen samples from oilseed rape (OSR) flowers (n=11), wildflowers**
 329 **from OSR margins (n=8) and WW margins (n=10), and collected by honeybees during OSR bloom (n=5) and after OSR bloom (n=5). OSR and wildflower**
 330 **pollens were collected in 3 farms, honeybee pollen samples were collected from hives sited on the vicinity of these farms. For the honeybee collected**
 331 **pollen, concentrations of the whole composite samples brought to the hives were used for the calculation of the means (i.e. one sample per hive was**
 332 **analysed).**

333 Overall these results reveal that pollen collected by honey bees are contaminated by similar
334 mixtures of pesticides as those present in wildflower pollen collected from OSR or WW field margins.
335 The most frequently detected (>28%) pesticides both in honey bee collected pollen and wildflower
336 pollen were thiamethoxam, thiacloprid, carbendazim, boscalid, spiroxamine, tebuconazole,
337 pyraclostrobin and trifloxystrobin. Carbendazim and spiroxamine were detected at concentrations
338 up to several hundreds of ng/g in some pollen samples. The totals for the mean measured
339 concentrations of pesticides in pollen were 166 ng/g from OSR, and for wildflowers sampled from
340 OSR and WW margins 78 and 25 ng/g respectively, and for honey bee pollen sampled during and
341 after the OSR blooming period 17 and 2.6 ng/g respectively (concentrations of the whole composite
342 pollen samples brought to the hives were used for the calculation of the means).

343 3.2 Neonicotinoid and fungicide levels in stored pollen and bumblebee individuals from nests placed
344 in rural and urban areas

345 The presence of neonicotinoids and fungicide mixtures in pollen and individual bumblebees sampled
346 from nests placed either in rural farmland or urban environments was determined. The range, mean
347 and median of the pesticide levels found are presented in Table 3.

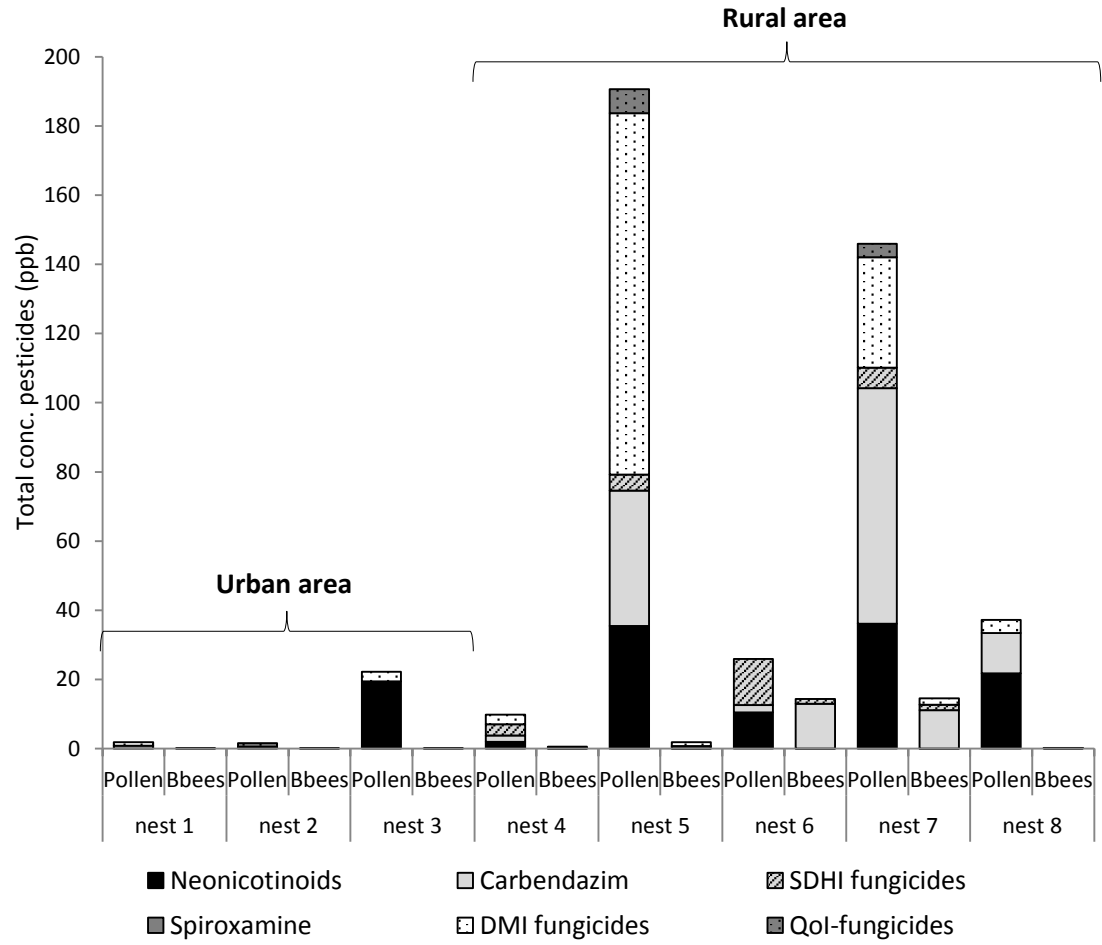
348 Pollen samples collected from the stores of individual nests placed in rural areas (n=5) contained
349 between 3 to 10 pesticide compounds. The most frequently detected compounds (35-100%) included
350 thiamethoxam, thiacloprid, carbendazim, boscalid, tebuconazole, flusilazole, metconazole and
351 trifloxystrobin and at concentrations up to 68 ng/g for carbendazim and 84 ng/g for flusilazole.
352 Imidacloprid, prochloraz and pyraclostrobin were also detected in 6% of the samples. Spiroxamine,
353 although frequently detected at high concentrations in OSR and wildflower margin pollen, was below
354 the MDL in bumblebee-collected pollen. The pollen from every nest was analysed as a whole, but the
355 analysis of identity and proportion of pollen types under light microscopy revealed that it comprised
356 a number of wildflower taxa with Rosaceae (*Crataegus monogyna*/*Malus* type) representing 42% in
357 average of the visited plants, and 32% on average coming from OSR flowers (Table S9). In bumblebee
358 individuals, the neonicotinoids thiamethoxam, acetamiprid and thiacloprid were detected at
359 concentrations below their MQLs. Carbendazim (up to 73 ng/g), boscalid (up to 10 ng/g), tebuconazole
360 (up to 5 ng/g), flusilazole and metconazole were detected above the MQLs in several individuals.
361 Carbendazim, boscalid, tebuconazole, flusilazole and metconazole were the most frequently detected
362 in 18-64% of individual bees. A comparison of the total pesticide concentrations in bumblebee and
363 pollen samples revealed large differences in pesticide contamination and exposure between each nest
364 (Figure 2).

365 **Table 3. The range, mean and median concentrations and frequency of detection of neonicotinoid and fungicide levels detected in stored pollen and in**
 366 **individual bumblebees sampled from nests sited in rural and urban landscapes.**

	Rural area								Urban area							
	Bumblebee pollen				Bumblebee				Bumblebee pollen				Bumblebee			
	Freq	Range	Mean	Median	Freq	Range	Mean	Median	Freq	Range	Mean	Median	Freq	Range	Mean	Median
%	ppb	ppb	ppb	%	ppb	ppb	ppb	%	ppb	ppb	ppb	%	ppb	ppb	ppb	
	n=5 / 5 nests				n= 28 / 5 nests				n= 3 / 3 nests				n= 15 / 3 nests			
Thiamethoxam	100	1.7 - 35	18	21	7	<0.03 - <0.09			0	<0.12			7	<0.03 - <0.09		
Clothianidin	0	<0.72			0	<0.48			0	<0.72			0	<0.48		
Imidacloprid	20	<0.36 - <1.1			0	<0.72			33	<0.36 - 20	6.5	<0.36	0	<0.72		
Acetamiprid	0	<0.02			7	<0.01 - <0.04			33	<0.02 - <0.07			0	<0.01		
Thiacloprid	60	<0.07 - 13	2.9	0.45	18	<0.02 - <0.07			0	<0.07			40	<0.02 - 0.17	0.02	<0.02
Carbendazim	100	1.8- 68	24	12	64	<0.05 - 73	4.6	0.25	67	<0.08 - 0.80	0.40	0.36	0	<0.05		
Carboxin	0	<0.12			0	<0.24			0	<0.12			0	<0.24		
Boscalid	80	<0.12 - 13	5.4	4.6	36	<0.24 - 9.8	0.60	<0.24	0	<0.12			0	<0.24		
Spiroxamine	0	<0.02			0	<0.05			0	<0.02			0	<0.05		
Silthiofam	0	<0.24			0	<0.24			0	<0.24			0	<0.24		
Triticonazole	0	<0.24			0	<0.48			0	<0.24			0	<0.48		
Epoxiconazole	0	<0.84			0	<0.96			33	<0.84 - 2.8	0.90	<0.84	0	<0.96		
Tebuconazole	80	<0.24 - 15	4.1	2.8	18	<0.12 - 5.2	0.20	<0.12	67	<0.24 - 1.1	0.40	<0.72	7	<0.12 - <0.36		
Flusilazole	40	<0.24 - 84	17	<0.24	14	<0.12 - 1.9	0.20	<0.12	0	<0.24			0	<0.12		
Prochloraz	20	<0.36 - 11	2.2	<0.36	0	<0.36			0	<0.36			0	<0.30		
Metconazole	40	<0.30 - 19	4.3	<0.30	4	<0.24 - 1.1	0.04	<0.24	0	<0.30			0	<0.24		
Pyraclostrobin	20	<0.24 - 2.4	0.50	<0.24	0	<0.24			33	<0.24 - 1.0	0.30	<0.24	0	<0.24		
Trifloxystrobin	40	<0.24 - 4.4	1.7	<0.24	0	<0.01			0	<0.24			0	<0.01		
Fluoxastrobin	20	<0.01 -<0.02			0	<0.24			0	<0.01			0	<0.24		
Piperonyl butoxide	0	<0.72			0	<0.24			0	<0.72			0	<0.24		

367 Pollen and bumblebees were collected from the same nests. Between 5 and 8 individuals per nest were analysed (except for one nest where only 3 workers
 368 were available). For the calculations of means and medians, all concentrations that were over the limits of detection (\geq MDL) but below the limits of
 369 quantification ($<$ MQL) were assigned the MDL value, whilst concentrations below the MDL were considered to be zero. ppb = ng/g wet weight of sample.
 370 Compounds highlighted in bold correspond to pesticides that were commonly found in pollen from both rural and urban areas.

371



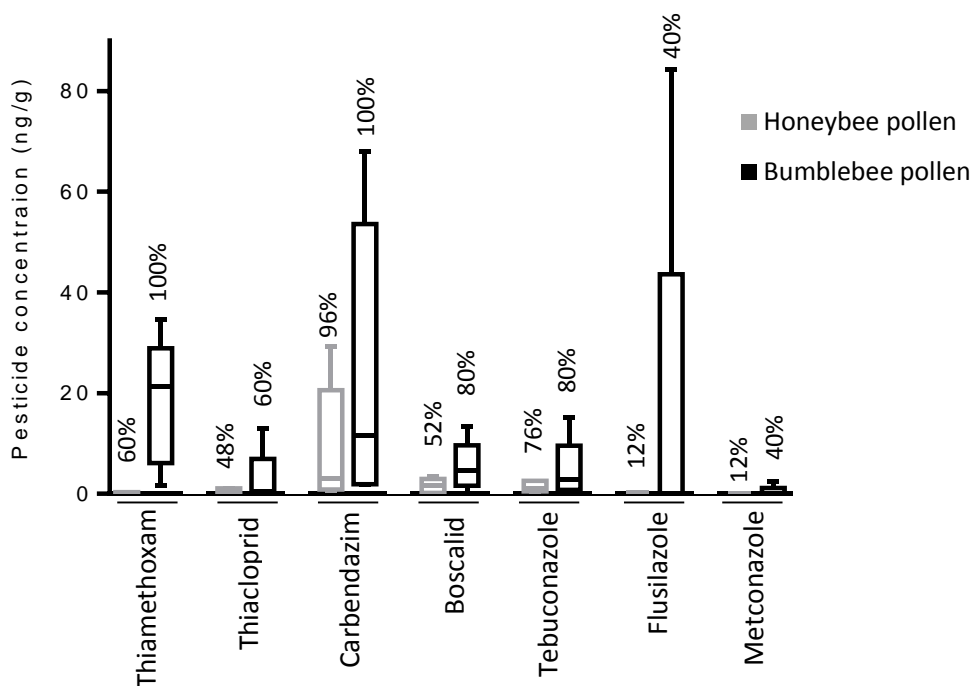
373

374 **Figure 2. The sum of the mean concentrations of neonicotinoids and fungicides in individual bumblebees (bbees) and collected pollen in nests sited in**
 375 **urban and rural areas.**

376 Concentrations of pesticides in pollen and bees sampled in urban areas (n=3) were much lower
 377 compared with rural areas (Figure 2). In nests placed in urban areas, six pesticides were detected in
 378 pollen collected by bumblebees; imidacloprid, acetamiprid, carbendazim, epoxiconazole,
 379 tebuconazole and pyraclostrobin. Imidacloprid was detected in pollen at up to 20 ng/g.
 380 Thiamethoxam, thiacloprid and tebuconazole were detected in bumblebee individuals at
 381 concentrations < 1 ng/g. Imidacloprid, carbendazim, tebuconazole and pyraclostrobin are the
 382 pesticides that were commonly found in pollen from both rural and urban areas.

383 A comparison of pollen collected by honey bees and bumblebees during the OSR bloom in rural
 384 landscapes revealed that many of the neonicotinoid and fungicide compounds which were present at
 385 concentrations > 1 ng/g were common to pollen collected by both bee species, but in this study
 386 exposure appeared to be much higher for bumblebees (Figure 3).

387 The insecticide synergist piperonyl butoxide was not detected in any of the pollen samples in this
 388 study.



389
 390
 391
 392 **Figure 3. Levels of thiamethoxam, thiacloprid, carbendazim, boscalid, tebuconazole, flusilazole and**
 393 **metaconazole in pollen samples collected by honeybee (n=5 beehives) and bumblebees (n=5**
 394 **nests).** Honeybee hives were placed in farms near OSR fields and the pollen was collected during the
 395 OSR bloom for 4 days using pollen traps. Concentrations of the whole composite samples brought to
 396 the hives were used for the calculation of the means. Bumblebee nests were placed in rural areas in
 397 arable landscapes and the pollen was collected after 4 weeks of free foraging in the field. The
 398 frequency of detection of neonicotinoid and fungicide are indicated above each box-and-whiskers-
 399 plots. The length of each box corresponds to the interquartile range, the upper and lower boundary

400 of the box representing 75th and 25th percentiles, respectively. The upper and lower whiskers
401 represent the maximum and the minimum values, respectively. The line in the box indicates the
402 median value.

403

404 **4. Discussion**

405 Debates over the impacts of pesticides on bees have tended to focus on the effects of specific
406 compounds or groups of compounds, with much attention in recent years on neonicotinoid
407 insecticides. However, it has recently become clear that honeybees are chronically exposed to
408 complex mixtures of pesticides (Johnson et al., 2012). Here, we show that both flowering crops and
409 nearby wildflowers are contaminated with a broad range of pesticides, and that this translates into
410 exposure of both honey bees and bumblebees to similar complex mixtures, with marked differences
411 in concentrations of pesticides in pollen collected by the two bee species. However, these differences
412 in concentrations between honeybee and bumblebee pollen must be tempered by the fact that the
413 bumblebee nests and the honeybee hives were placed in different rural areas and by the fact that
414 honeybee pollen was gathered for 4 days using traps, whereas bumblebees foraged for 4 weeks before
415 the pollen was collected in the nests. Nevertheless, it is likely that the pollen sample collected by
416 bumblebees was gathered in the previous two-three days as they keep low storage levels to avoid
417 theft of honey and pollen by mammals (Heinrich 2004).

418 Our data show that the pollen of oilseed rape crops is contaminated with a broad range of pesticides,
419 notably spiroxamine, carbendazim, the neonicotinoids thiamethoxam and clothianidin, a range of DMI
420 fungicides and trifloxystrobin. Other fungicides, i.e. boscalid, pyraclostrobin and fluoxastrobin were
421 also present but less frequently detected. Broadly similar cocktails, at generally slightly lower
422 concentrations, were found in hand-collected pollen from wildflowers in arable field margins. It should
423 be noted that this is not an exhaustive list of the pesticides present; in particular we did not screen for
424 pyrethroids because these require an entirely different analytical approach, but these were used on
425 the farms we studied.

426 Some of the neonicotinoids and fungicides that we have detected in honeybee collected pollen had
427 already been detected in similar pollen samples in other studies, although this is the first study
428 providing data in bee pollen for this mixture of pesticides in UK. It should be noted however that these
429 studies used composite pollen samples (as opposed to pollen from individual species here) and
430 therefore provide less information on the variability of exposure levels. In pollen samples from honey
431 bee colonies in western France, carbendazim and flusilazole were detected at concentrations up to

432 2595 ng/g and 52 ng/g respectively (as opposed to 120 and 6.1 ng/g respectively in our study)
433 (Lambert et al., 2013). Higher concentrations of thiacloprid, imidacloprid, carbendazim,
434 trifloxystrobin, boscalid, tebuconazole, pyraclostrobin and trifloxystrobin were also observed in
435 honeybee pollen collected in hives from North America (up to 962 ng/g for boscalid) (Mullin et al.,
436 2010) but their frequencies were generally much lower than those detected in this study. Overall, our
437 results and these studies indicate that these mixtures of insecticides and fungicides appear ubiquitous
438 in pollen samples and that even higher concentrations than the ones observed in our study can be
439 encountered.

440 Honey bees and the bumblebee *Bombus terrestris* are both highly polylectic in their flower visits; both
441 are regular visitors to OSR flowers (Cresswell and Osborne 2004), but both taxa also visit a broad range
442 of wildflowers present in field margins and hedgerows, gardens, and uncropped areas, though the two
443 species exhibit different floral preferences (Wood et al., 2015). We would thus expect both species to
444 be exposed to the chemicals we found in pollen of the crop and wildflowers, and indeed this was the
445 case. It is worth noting that for both species, pollen from hawthorn represents a major part of the
446 collected pollen (up to 87%) and that the pollen from hawthorn collected by honeybees was often
447 contaminated by several pesticides (up to 4) and notably at concentrations up to 33 ng/g for
448 carbendazim.

449 For pollen collected by honeybees, the major pesticide contaminants were (in declining order of mean
450 concentration) carbendazim, boscalid, spiroxamine, tebuconazole and trifloxystrobin, with small
451 amounts of the neonicotinoids thiacloprid, imidacloprid and thiamethoxam. Overall, the
452 concentrations tend to be lower than in the crop or adjacent wildflowers, likely to be because the bees
453 are also collecting pollen from uncontaminated wildflowers distant from arable fields, diluting the
454 overall concentration returning to the hive. There was a notable reduction in the concentrations of
455 neonicotinoids and fungicides detected in honey bee pollen collected after OSR blooming, presumably
456 because the bees are no longer feeding on treated crops but also perhaps because of ongoing
457 biodegradation and photolysis of pesticide residues in the environment as summer progresses
458 (Bonmatin et al., 2015; Gupta et al., 2008).

459 Concentrations of pesticides in pollen collected by bumblebees were markedly different to those for
460 pollen collected by honeybees during the OSR bloom (Figure 3). The major contaminants were
461 carbendazim, thiamethoxam and flusilazole. The high levels of thiamethoxam are particularly
462 noteworthy, for this is an insecticide of high toxicity to bees. Experimental studies such as Whitehorn
463 et al. (2012) which describe severe impacts of neonicotinoids on bumblebees have been criticised for
464 using unrealistically high concentrations of pesticide (in this example 6 ng/g of imidacloprid) (Carreck

465 and Ratnieksi 2014). Our data suggest that real-world exposure may often be much higher than this,
466 for the mean concentration of thiamethoxam in our samples from 5 nests located in farmland was 18
467 ng/g, and one of the nests located in urban environment showed more than 19 ng/g for imidacloprid.
468 It has also been demonstrated that there are synergies between neonicotinoids and DMI fungicides
469 such as flusilazole (Iwasa et al., 2004; Schmuck et al., 2003), so the presence of both compounds at
470 high concentrations in pollen stores of bumblebees is a cause for concern.

471 Recently, Ründlof et al. (2015) found that bumblebee colonies were adversely affected by proximity
472 to fields of OSR treated with clothianidin (the major bioactive metabolite of thiamethoxam), but that
473 honeybees showed no significant harm, at least within one season. Our results suggest an explanation
474 for this disparity; bumblebees may simply be exposed to the pesticide more, perhaps because of a
475 greater propensity to collect OSR pollen (i.e. proportion of OSR pollen was 10% in average for
476 honeybees as opposed to 32% in average for bumblebees). It may also be because bumblebees tend
477 to forage over shorter distances compared to honeybees (Knight et al., 2005), which may mean that
478 there is less dilution of pesticide residues coming in to the nest when these are located in the vicinity
479 of arable lands. However, it should be noted that our data set is small, and that honeybee hives and
480 bumblebee colonies were not placed in exactly the same localities. They were also sampled in different
481 ways; honeybee pollen was collected from returning bees using a pollen trap, whereas pollen traps
482 are not effective for bumblebees and hence pollen was taken from stores inside the nest. Further
483 research is clearly needed to confirm whether bumblebees really are more prone to collect pollen
484 contaminated with pesticides, and if so, why.

485 Our sampling was conducted in the spring and summer of 2013. Since then, a moratorium on the use
486 of neonicotinoids as seed dressings on flowering crops has come into effect in the EU (though some
487 individual countries have granted derogations for continued use). It would be fascinating to repeat
488 our work to examine whether contamination of wildflowers and bee pollen with neonicotinoids has
489 dropped as a result.

490 In contrast to rural areas, there were generally few pesticide residues in pollen collected by
491 bumblebee colonies in the 3 nests placed in urban areas. Imidacloprid was the biggest contaminant,
492 and the only neonicotinoid detected. To our knowledge, these are the first data pertaining to exposure
493 of bees to pesticides in urban environments, and a more extensive study is needed to determine
494 whether pesticide exposures are much lower in these areas. While pesticide usage data in the UK is
495 available for farmland, no data are publicly available on sales or usage of pesticides by gardeners and
496 local authorities, and very little information is available on likely levels of contamination of ornamental
497 plants with pesticides, so we can only speculate as to the source of this exposure. Imidacloprid was

498 widely sold in the UK as a garden insecticide in the past, but has been largely replaced by thiacloprid
499 and acetamiprid in recent years (D.G. pers. obs.). It is unclear whether the imidacloprid found in our
500 samples is due to persistent residues from past use, or due to ongoing environmental contamination
501 from other sources – for example imidacloprid is the active ingredient in formulations widely used for
502 ant control (e.g. “Maxforce Quantum”, Bayer Crop Science) and for flea control on domestic animals
503 (e.g. “Advantage”, Bayer Crop Science).

504 It has previously been found that bumblebee populations in gardens are higher than those in farmland
505 (Goulson et al., 2010; Osborne et al., 2008), and our results may in part explain why – because they
506 could be exposed to fewer pesticides. However, they also probably have access to a greater
507 abundance and diversity of floral resources in gardens, and without further experimental
508 manipulations we cannot determine which of these factors is most important.

509 Screening of whole bees for pesticides detected generally low concentrations, compared to pollen
510 samples (Table 3), although a range of DMI fungicides were found at concentrations exceeding 1 ng/g
511 in some samples, and carbendazim was found at a mean concentration of 4.6 ng/g in bumblebees
512 from rural areas. There were also detectable traces of the neonicotinoids thiamethoxam, acetamiprid
513 and thiacloprid in some bees. For practical reasons, bumblebee pollen and bumblebee individuals
514 were collected at different times (individuals were collected 6 weeks after the pollen was collected,
515 i.e. after the OSR bloom) and this could partially explain the lower concentrations observed for some
516 pesticides in bumblebees. Despite this, it seems likely that pesticides are metabolised at varying rates
517 once consumed by bees; for instance, it has been shown that bumblebees can clear imidacloprid from
518 their body after 2 days of exposure (Cresswell et al., 2014) and a half-life of 5 hours has been recorded
519 for honey bees (Suchail et al., 2004). A recent study has revealed that detoxification of the xenobiotic,
520 nicotine, in bees, was associated with increased energetic investment and antioxidant and heat shock
521 response (du Rand et al., 2015). The process of detoxifying an array of xenobiotics arising from
522 exposure to agrochemicals and secondary plant products may result in metabolic stress and increased
523 susceptibility of the bee to pathogens and disease (Goulson et al., 2015).

524 It is notable that the bulk of pesticides found in both honeybee pollen and bumblebee pollen were
525 fungicides, particularly carbendazim, boscalid, tebuconazole, flusilazole, metaconazole,
526 pyraclostrobin and trifloxystrobin. Although fungicides have generally low toxicity to bees (Johnson
527 2015), little is understood about the impacts they may have on beneficial fungi commonly found in
528 stored pollen (bee bread). Classes of fungicides commonly found in bee pollen in our study (boscalid,
529 DMIs and quinone outside inhibitors, QoIs) have been reported to be fungicidal against 12 fungal
530 species isolated from bee bread (Yoder et al., 2012). Bee bread is produced by fungal fermentation of

531 stored pollen and is important food for honey bee larvae. Alterations in the diversity of fungi may
532 affect food value and also allow pathogenic fungi such as the etiological agent of chalkbrood disease,
533 *Ascosphaera apis*, to thrive in the hive, thus affecting colony performance (Yoder et al., 2013).

534 In summary, our study confirms that bees foraging in arable farmland are exposed to a complex
535 cocktail of neonicotinoid insecticides and fungicides in the pollen they collect, with exposure of
536 bumblebee colonies being far higher than that of honeybees. While quantifying realistic levels of
537 exposure via pollen as we have done here is an important step forwards, we did not examine exposure
538 via nectar, which we intend to address in future work. A major challenge which has yet to be tackled
539 is attempting to understand what effects simultaneous exposure to multiple pesticides has upon bees
540 in the field.

541 **Acknowledgments**

542 We are grateful to the Soil Association (Bristol, UK) for part funding of this work, and to the farmers
543 for allowing us to collect samples on their farms and for sharing their pesticide usage data.

544 **Conflict of Interest**

545 The authors declare that they have no conflict of interest.

546 **Statement on animal ethical care**

547 The work reported here conforms to the regulatory requirements for animal experimentation in the
548 UK. No ethics approval was required for this study. Honeybee hives and bumblebee nests were housed
549 on private land for which research permission was granted by the owners. This study did not involve
550 endangered or protected species.

551

552 **References**

- 553 Bingham, G., Gunning, R.V., Delogu, G., Borzatta, V., Field, L.M., Moores, G.D., 2008. Temporal
554 synergism can enhance carbamate and neonicotinoid insecticidal activity against resistant
555 crop pests. *Pest Manag Sci.* 64, 81-85.
- 556 Bonmatin, J.M., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C., et al., 2015.
557 Environmental fate and exposure; neonicotinoids and fipronil. *Environ Sci Pollut R.* 22, 35-
558 67.
- 559 Botías, C., David, A., Horwood, J., Abdul-Sada, A., Nicholls, E., Hill, E., et al., 2015. High prevalence of
560 neonicotinoid residues in wildflowers, a potential route of chronic exposure for bees.
561 *Environ Sci Technol.* submitted

562 Carreck, N.L., Ratnieksi, F.L.W., 2014. The dose makes the poison: have "field realistic" rates of
563 exposure of bees to neonicotinoid insecticides been overestimated in laboratory studies? *J*
564 *Apicult Res.* 53, 607-614.

565 Cresswell, J.E., Osborne, J.L., 2004. The effect of patch size and separation on bumblebee foraging in
566 oilseed rape: implications for gene flow. *J Appl Ecol.* 41, 539-546.

567 Cresswell, J.E., Robert, F.X., Florance, H., Smirnoff, N., 2014. Clearance of ingested neonicotinoid
568 pesticide (imidacloprid) in honey bees (*Apis mellifera*) and bumblebees (*Bombus terrestris*).
569 *Pest Manag Sci.* 70, 332-337.

570 David, A., Botías, C., Abdul-Sada, A., Goulson, D., Hill, E.M., 2015. Sensitive determination of mixtures
571 of neonicotinoid and fungicide residues in pollen and single bumblebees using a scaled down
572 QuEChERS method for exposure assessment. *Anal Bioanal Chem.* In press DOI
573 10.1007/s00216-015-8986-6.

574 Demske, D., Tarasov, P.E., Nakagawa, T., Members, S.P., 2013. Atlas of pollen, spores and further non-
575 pollen palynomorphs recorded in the glacial-interglacial late Quaternary sediments of Lake
576 Suigetsu, central Japan. *Quatern Int.* 290, 164-238.

577 du Rand, E.E., Smit, S., Beukes, M., Apostolides, Z., Pirk, C.W.W., Nicolson, S.W., 2015. Detoxification
578 mechanisms of honey bees (*Apis mellifera*) resulting in tolerance of dietary nicotine. *Sci Rep-
579 Uk.* 5,

580 Fairbrother, A., Purdy, J., Anderson, T., Fell, R., 2014. Risks of neonicotinoid insecticides to honeybees.
581 *Environ Toxicol Chem.* 33, 719-731.

582 Goulson, D., 2013. REVIEW: An overview of the environmental risks posed by neonicotinoid
583 insecticides. *J Appl Ecol.* 50, 977-987.

584 Goulson, D., Lepais, O., O'Connor, S., Osborne, J.L., Sanderson, R.A., Cussans, J., et al., 2010. Effects of
585 land use at a landscape scale on bumblebee nest density and survival. *J Appl Ecol.* 47, 1207-
586 1215.

587 Goulson, D., Nicholls, E., Botias, C., Rotheray, E.L., 2015. Bee declines driven by combined stress from
588 parasites, pesticides, and lack of flowers. *Science.* 347, 1435-+.

589 Gupta, S., Gajbhiye, V.T., Gupta, R.K., 2008. Effect of light on the degradation of two neonicotinoids
590 viz acetamiprid and thiacloprid in soil. *Bull Environ Contam Toxicol.* 81, 185-189.

591 Heinrich, B. *Bumblebee economics.* Cambridge, Mass.: Harvard University Press; 2004.

592 Human, H., Brodschneider, R., Dietemann, V., Dively, G., Ellis, J.D., Forsgren, E., et al., 2013.
593 Miscellaneous standard methods for *Apis mellifera* research. *J Apicult Res.* 52,

594 Iwasa, T., Motoyama, N., Ambrose, J.T., Roe, R.M., 2004. Mechanism for the differential toxicity of
595 neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Prot.* 23, 371-378.

596 Johnson, R.M., 2015. Honey Bee Toxicology. *Annual Review of Entomology*, Vol 60. 60, 415-434.

597 Johnson, R.M., Ellis, M.D., Mullin, C.A., Frazier, M. *Pesticides and Honey Bee Toxicity in the United
598 States.* Boca Raton: Crc Press-Taylor & Francis Group; 2012.

599 Khan, H.A.A., Akram, W., Iqbal, J., Naeem-Ullah, U., 2015. Thiamethoxam Resistance in the House Fly,
600 *Musca domestica* L.: Current Status, Resistance Selection, Cross-Resistance Potential and
601 Possible Biochemical Mechanisms. *PLoS One.* 10,

602 Kirk, W.D.J. *A Colour Guide to Pollen Loads of the Honey Bee.* Treforest: International Bee Research
603 Association; 2006.

604 Knight, M.E., Martin, A.P., Bishop, S., Osborne, J.L., Hale, R.J., Sanderson, A., et al., 2005. An
605 interspecific comparison of foraging range and nest density of four bumblebee (*Bombus*)
606 species. *Mol Ecol.* 14, 1811-1820.

607 Lambert, O., Piroux, M., Puyo, S., Thorin, C., L'Hostis, M., Wiest, L., et al., 2013. Widespread occurrence
608 of chemical residues in beehive matrices from apiaries located in different landscapes of
609 Western France. *PLoS One.* 8, e67007.

610 Moore, P.D., Webb, J.A., Collinson, M.E. *Pollen Analysis.* Oxford: Wiley-Blackwell; 1991.

611 Mullin, C.A., Chen, J., Fine, J.D., Frazier, M.T., Frazier, J.L., 2015. The formulation makes the honey bee
612 poison. *Pest Biochem Physiol.* 120, 27-35.

613 Mullin, C.A.,Frazier, M.,Frazier, J.L.,Ashcraft, S.,Simonds, R.,Vanengelsdorp, D., et al., 2010. High
614 levels of miticides and agrochemicals in North American apiaries: implications for honey bee
615 health. PLoS One. 5, e9754.

616 Osborne, J.L.,Martin, A.P.,Shortall, C.R.,Todd, A.D.,Goulson, D.,Knight, M.E., et al., 2008. Quantifying
617 and comparing bumblebee nest densities in gardens and countryside habitats. J Appl Ecol.
618 45, 784-792.

619 Ründlof, M.,Andersson, G.K.,Bommarco, R.,Fries, I.,Hederstrom, V.,Herbertsson, L., et al., 2015. Seed
620 coating with a neonicotinoid insecticide negatively affects wild bees. Nature. 521, 77-80.

621 Sanchez-Bayo, F.,Goka, K., 2014. Pesticide Residues and Bees - A Risk Assessment. PLoS One. 9,
622 Sawyer, R. Pollen Identification for Beekeepers. Cardiff: University College Cardiff Press; 1981.

623 Schmuck, R.,Stadler, T.,Schmidt, H.W., 2003. Field relevance of a synergistic effect observed in the
624 laboratory between an EBI fungicide and a chloronicotiny insecticide in the honeybee (*Apis*
625 *mellifera* L, Hymenoptera). Pest Manag Sci. 59, 279-286.

626 Suchail, S.,Debrauwer, L.,Belzunces, L.P., 2004. Metabolism of imidacloprid in *Apis mellifera*. Pest
627 Manag Sci. 60, 291-296.

628 Thompson, H.M.,Fryday, S.L.,Harkin, S.,Milner, S., 2014. Potential impacts of synergism in honeybees
629 (*Apis mellifera*) of exposure to neonicotinoids and sprayed fungicides in crops. Apidologie.
630 45, 545-553.

631 Whitehorn, P.R.,O'Connor, S.,Wackers, F.L.,Goulson, D., 2012. Neonicotinoid Pesticide Reduces
632 Bumble Bee Colony Growth and Queen Production. Science. 336, 351-352.

633 Wood, T.J.,Holland, J.M.,Goulson, D., 2015. Pollinator-friendly management does not increase the
634 diversity of farmland bees and wasps. Biological Conservation. 187, 120-126.

635 Yoder, J.A.,Hedges, B.Z.,Heydinger, D.J.,Sammataro, D.,DeGrandi-Hoffman, G. Differences Among
636 Fungicides Targeting Beneficial Fungi Associated with Honey Bee Colonies. Boca Raton: Crc
637 Press-Taylor & Francis Group; 2012.

638 Yoder, J.A.,Jajack, A.J.,Rosselot, A.E.,Smith, T.J.,Yerke, M.C.,Sammataro, D., 2013. Fungicide
639 Contamination Reduces Beneficial Fungi in Bee Bread Based on an Area-Wide Field Study in
640 Honey Bee, *Apis mellifera*, Colonies. J Toxicol Env Heal A. 76, 587-600.

641 Zhu, W.Y.,Schmehl, D.R.,Mullin, C.A.,Frazier, J.L., 2014. Four Common Pesticides, Their Mixtures and
642 a Formulation Solvent in the Hive Environment Have High Oral Toxicity to Honey Bee Larvae.
643 PLoS One. 9, 11.

644

645

646