Novel indices reveal that pollinator exposure to pesticides varies across biological compartments and crop surroundings

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Highlights

- We use new indices to summarise big datasets on pesticide exposure of three species of bees
- Novel indices are calculated using Item Response Theory (IRT) models
- The indices are linked to the number of pesticides rather than the active ingredients
- Matrices collected from apple orchards are exposed to a higher number of pesticides than matrices collected from oilseed rape crops
- Pollen related matrices contained more pesticides than were found in nectar and on the bees themselves

Abstract.

Declines in insect pollinators have been linked to a range of causative factors such as disease, loss of habitats, the quality and availability of food, and exposure to pesticides. Here, we analysed an extensive dataset generated from pesticide screening of foraging insects, pollen-nectar stores/beebread, pollen and ingested nectar across three species of bees collected at 128 European sites set in two types of crop. In this paper, we aimed to (i) derive a new index to summarise key aspects of complex pesticide exposure data and (ii) understand the links between pesticide exposures depicted by the different matrices, bee species and crops. We found that summary indices were highly correlated with the number of pesticides detected in the related matrix but not with which pesticides were present. Matrices collected from apple orchards generally contained a higher number of pesticides (7.6 pesticides per site) than matrices from sites collected from oilseed rape crops (3.5 pesticides), with fungicides being highly represented in apple crops. A greater number of pesticides were found in pollen-nectar

66 stores/beebread and pollen matrices compared with nectar and bee body matrices. Our
67 results show that for a complete assessment of pollinator pesticide exposure, it is necessary
68 to consider several different exposure routes and multiple species of bees across different
69 agricultural systems.

70

71 **Keywords**

72 Item Response Theory. Bumble bee. Osmia. Apple orchards. Oilseed rape.

73

1. Introduction

Declines in species of both managed and wild pollinators has been repeatedly documented [1] in Europe [2], the US [3], Canada [4], Asia [5] and to some extend in South-America [6] and Africa [7]. Managed bees such as honeybees (*Apis mellifera*) [8] and wild bees [9, 10] are the most important group of pollinators in Europe and other regions of the world (IPBES 2016). A range of factors have been suggested to explain losses of bees such as diseases [11, 12], loss of habitats [13, 14], the quality and availability of food [15, 16] and exposure to pesticides [17, 18]. The way bees are exposed to pesticides is variable and depends mainly on the type of pesticide [19, 20], their purpose of use (which is related to the application mode i.e. spray, soil treatment, trunk injection), [21] and on the ecology of species [22, 23]. Application timing (pre-bloom *versus* at-bloom) has logically dramatic impacts on exposure levels for pollinators feeding on nectar and pollen from flowers [18]. Several techniques have been developed to limit this exposure such as microencapsulated compounds and seed coated insecticides with systemic properties [24]. Bees can also be exposed to pesticides through water consumption [25, 26], pesticide contact [27], air [19, 28, 29] and, in the case of managed bees, the use of veterinary products [30, 31]. However, dietary consumption is the major route of exposure [18].

Honeybees produce large quantities of honey from collected nectar. In addition, for storage purposes, after collection, pollen grains are processed into beebread. This term usually refers to honeybee pollen stores, as beebread is pollen with added nectar and enzymes [32] and stored in frames made of beeswax. For other bee species, however, any substance consisting predominantly of stored pollen will be referred to as pollen-nectar stores in this paper.

Previously, pesticide residues have been documented in nectar [18], honey [33], pollen collected on flowers [19], honeybee pollen pellets collected with traps [34], honeybee beebread [35], wax [36] and honeybees themselves [37]. However, the majority of exposure studies describe the contamination of one or two matrices at the same time [38]. To our knowledge, our study is the first to present results across pesticides in pollen collected from flowers and from pollen pellets, in pollen-nectar stores and beebread, in nectar regurgitated from honeybees and from other bee species and from bee bodies, collected at the same time in the same site. In an attempt to better understand the exposure route of three bee species (*Apis mellifera*, *Bombus terrestris* and *Osmia bicornis*), we assessed pesticides in each of these matrices at the same time in 128 sites set in two types of crops (apple orchards, oilseed rape) across Europe. To our knowledge, this dataset is one of the most extensive datasets of bee exposure to pesticides currently available.

As the number of pesticides measured in the different matrices and for each site was very large, it was necessary to synthetise this complex information. The construction of such indices, that are able to summarise information for all pesticides detected at a site, is of paramount interest. Such an index can be used, for instance, for investigating the links between the different matrices under study or in structuring model equations to explore the role of stresses on bee population dynamics. A classic way to summarise pesticide information is to calculate the richness (i.e., the number of pesticides detected in a given sample), or the abundance (i.e., the total quantity of pesticides detected in a given sample) [39]. However, these simple calculations do not capture information on pesticide variability across the samples. In this paper, we propose to apply an original method, namely Item Response Theory (IRT) models to calculate an index that includes as much variability as possible while being easily interpretable.

120 The IRT models build such indices, each being associated with a matrix (i.e., pollen-nectar
121 stores or beebread, pollen, nectar and foragers from different species and flowers) and a crop
122 (i.e., apple orchards, oilseed rape). We also propose a method to interpret these indices
123 (section 3.1). In a second step, the links between all these indices are studied (section 3.2).
124 Results are discussed in the context of the existing literature (section 4).

125

126 ## 2. Materials and methods

127 ### 2.1. Samples collection in PoshBee site network

128 Within the H2020 project 'PoshBee' (www.PoshBee.EU), a site network for assessing exposure
129 of bees to chemical, nutritional, and pathogen stressors was established in 2019 [40]. Data
130 were collected at 128 sites across eight participating countries (Estonia, Germany, Ireland,
131 Italy, Spain, Sweden, Switzerland and the United Kingdom) situated in either apple orchards
132 or oilseed rape crops. At each site, three honeybee colonies, three trap nests seeded with
133 male and female cocoons of *Osmia bicornis* (solitary bee) and three *Bombus terrestris*
134 (bumblebee) colonies were installed following the PoshBee protocols [40].

135 At each site, various matrices were collected from all colonies and nests in equal proportions,
136 pooled per species and subsequently sent for pesticide residues analyses in different
137 laboratories [41]. If field constraints prevented the collection of equal proportions, acceptable
138 differences between colony/nest were limited to a maximum of 30%. If one colony/nest did
139 not produce the quantity required, the quantities from the remaining two were increased in
140 order to reach the total quantity required. The sampling of each matrix was performed only
141 once for each species at each site generally on the same day. Depending on the matrix,
142 sampling was performed either during or towards the end of the flowering period to be

143 consistent with biological cycles of bees (Figure A1 and Figure A2, in supplementary material).
144 At each site, *A. mellifera* and *B. terrestris* adults were collected alive. Bees were gently pressed
145 at the two first abdominal segments on the crop (honey sack) until a drop of nectar was
146 regurgitated between the bee mandibles. Nectar was collected was pooled for each species
147 to produce one sample per species for each site for pesticide analysis.

148 The matrices listed in Figure A1 were sampled and subsequently analysed for determination
149 and quantification of pesticide residues. Due to the behavior and limited success of solitary
150 bees in the wild, it was not possible to obtain sufficient numbers of *O. bicornis* bees or
151 amounts of regurgitated nectar to perform analyses for pesticide residues on these matrices
152 (Table 1).

153

154 **2.2. Analytical methods for pesticide determination and quantification**

155 Four different laboratories analysed the samples to identify and quantify pesticide residues.
156 Each laboratory was in charge of a specific matrix and had a specific developed and validated
157 method with LC-MS/MS or GC-MS/MS. The different analytical methods were detailed for
158 pollen-nectar stores and beebread [31], nectar (Martel et al, submitted), bees [42] and pollen
159 from flowers and from traps. This resulted in five different lists of pesticides depending on
160 matrices. However, 64 common pesticides were selected at the beginning of PoshBee based
161 on agrochemicals applied on crops at the European level to enable comparison between
162 matrices. The index calculation was not restricted to these 64 pesticides. Indeed, if a pesticide
163 was detected in only one matrix, it contributed to increase the exposure in the site where it
164 was detected. As a consequence, the indices' values increased. At the end, 267 pesticides were
165 screened for in pollen-nectar stores and beebread, 373 pesticides in foragers, 85 pesticides in

166 nectar, 336 pesticides in pollen from *A. mellifera* traps and 300 pesticides in pollen from
167 flowers.

168 A minimum quantity was required to perform laboratory analysis. This requirement was not
169 always met due to field constraints. Thus, results were missing for some sites or matrices. At
170 the end, 319 pollen-nectar store/beebread samples, 253 forager samples, 251 nectar samples,
171 117 *A. mellifera* pollen-trap samples and 60 flower pollen samples were analysed (Table 1).

172 Table 1 – Overview of the number of sites sampled and analysed, the number of pesticides screened and detected
173 in each matrix for each species and crop corresponding to the 18 datasets included in the indices calculation. The
174 percentages of sites with analysed samples were compared to the theoretical number of samples according to
175 the protocol (=64 samples for each matrix, i.e., 8 sites × 8 countries). *A. m*: *Apis mellifera*. *B. t*: *Bombus terrestris*.
176 *O. b*: *Osmia bicornis*. APP: apple. OSR: oilseed rape. *Apis*: pollen collected with pollen traps set up on *A. mellifera*
177 colonies.

178

179 The quality and consistency of all the analytical results was automatically controlled in a
180 database designed for this purpose (named Poshbase) enabling the collection of 18 datasets
181 corresponding to the matrices across the three bee species (Table 1).

182 The theoretical number of sites under study was 64 for a given matrix and crop (Table 1).
183 However for various reasons (i.e. quantity of sampled matrix not sufficient for subsequent
184 laboratory analysis, difficulty to retrieve matrix from the field due to weather conditions or
185 scarce quantity), the actual number of sites in the statistical analysis was reduced. The largest
186 reduction was observed for the pollen collected directly on flowers in apple orchards (N=26)
187 and oilseed rape (N=34). The number of sites with at least one pesticide detected in a matrix
188 varied from 100% in beebread from honeybee colonies in apple orchards or oilseed rape and
189 in pollen-nectar stores from solitary bees' nests in oilseed rape crops for instance, to 33% in

190 bumblebee foragers in oilseed rape crops. Between 11 (in bumblebees in oilseed rape crops)
191 and 98 (in honeybee beebread collected in colonies in apple orchards) pesticides were
192 detected in any given matrix, representing between 3% and 37% of the pesticides screened
193 for.

194 As the calculation of the indices was intended to give the best discrimination between sites,
195 only pesticides detected in at least one site were taken into account. Thus, each dataset used
196 for the statistical analysis was of dimension N × P (Table 1; e.g. for *Beebread.Apis* and for apple
197 orchards, P=98 pesticides were detected and measured in N=62 sites) and included the
198 quantification of each pesticide in each site. More precisely for a given site, a given pesticide
199 and a given matrix, the following rules were applied: the LOQ (limit of quantification, the
200 pesticides detected below this value cannot be quantified) was used for values between the
201 LOD (limit of detection; below this value, the pesticides cannot be detected with sufficient
202 confidence) and the LOQ, and quantified values were kept in cases of values higher than LOQ.
203 As the data had many zeros (i.e., non-detected pesticides), the calculation of the indices was
204 based on binary data: 0 was used if the value was inferior to LOD and 1 was used otherwise.
205 However, the index's interpretation was based on raw quantified values.

206

207 **2.3. Statistical analyses**

208 Our aim was to summarise and interpret the large amount of information available in each
209 dataset. For this purpose and in a first step, 18 indices were built, one for each matrix and
210 each crop. The objective was to reduce the dimensionality of the datasets to characterise the
211 site exposure to pesticides in a unidimensional and interpretable index. Subsequently, each
212 index was interpreted according to the pesticides detected. Finally, and for each crop, the links

between the nine indices were studied with a Principal Component Analysis as a summary of correlation matrix (Figure 1).

Figure 1 - The overall statistical procedure for a given crop (apple orchard or oilseed rape) for the nine matrices across the three bee species (*Pollen.Flower*, *Nectar.Apis*, *Apis*, *Pollen.Apis*, *Beebread.Apis*, *Nectar.Bombus*, *Bombus*, *Pollen-nectar stores.Bombus*, *Pollen-nectar stores.Osmia*). The map is from Hodge et al. 2022. IRT: Item Response Theory. PCA: Principal Component Analysis.

Calculation of indices. Initially developed in the psychology framework, the Item Response Theory (IRT) models aim at building a unidimensional scale (= latent trait = index), from different items that measure this trait [43, 44]. The IRT concept was translated as to whether a site exhibited a given pesticide or if the pesticide was absent from the site. The more pesticide were recorded the i^{th} site was, the higher its index value, denoted θ_i.

For a given pesticide j, the two parameters to be estimated in the model were the mean exposure level of a site (a_j) and the specific exposure level of a site (b_j), fitted with an EM algorithm (Chalmers, 2012). The exposure level (measured here as the number of detected pesticides per site) was the level a site should have, to have 50% chance to exhibit a pesticide. The specific exposure level represented how well the item (i.e. pesticide) separated sites with high exposure scores from sites with low exposure scores. In theory, most, if not all pesticides, should have a positive specific exposure level: the more exposed a site was, the more likely it was to detect a given pesticide. For this purpose, the following two-parameter logistic model was applied. Let $P(X_{i,j}|\theta_i)$ be the probability that the site i exhibited the pesticide j given its exposure level, such as:

$$P(X_{i,j}|\theta_i) = \frac{1}{1+e^{-a_j(\theta_i - b_j)}}$$ for the j^{th} pesticide and the i^{th} site (i=1, …, 64)

With a_j the exposure level, b_j the site-discrimination and θ_i the level of exposure at site i.

For several pesticides under study, the previous model was adapted: all the pesticides were included and then selected through a backward selection algorithm applied to filter out non-interpretable pesticides. To maximize the statistical significance of the two parameters (a_j and b_j), a double control on each step of the algorithms was implemented: (i) a stepwise loop stopped if there were no more pesticides with a negative discrimination, or (ii) if the performance criterion of the model (=Akaike information criterion, AIC) stopped decreasing. At the end, only pesticides with a positive discrimination were retained. In addition, the stability of the selection was tested with a leave-one-out cross validation, both on sites and pesticides. In summary, using the index was relevant when the information on the pesticide detection was fragmented between different pesticides (see the discussion for details).

Interpretation of indices. The index was calculated on pesticide presence or absence to have robust calculations and deal with the many zeros. However, as the interpretation was not based on more robust statistical tests, the quantities of pesticides from the raw quantified data were used (Table 2). For a given matrix and a given crop, the pesticides, as well as countries, that most contributed to the index were highlighted and interpreted. For this purpose, all the available sites were clustered by means of a Hierarchical Clustering Analysis applied to each index value [45]. Then, the pesticides that were significantly over-represented in a cluster compared to the mean were highlighted [46]. Similarly, under-represented pesticides compared to the mean could also be identified; they were detailed only in Table 2 for the example and interpretation. Two supplementary variables (i.e., number of pesticides and country) were also taken into account. Sites of a given country that were over- or under-represented in a cluster compared to the mean were also highlighted. Consequently, the

interpretation of presence/absence of sites from a given country compared to sites from other countries was possible (see Table 2). It is worth noting that the number of sites per country (N=8 sites) did not allow the extrapolation of results to the whole country. Indeed, the site network was not designed to be representative of countries, but rather to be representative of these crop landscapes in the European territory.

Links between indices. For a given crop (apple or oilseed rape), the links between the nine indices – related to the different matrices – were studied with a Principal Component Analysis (PCA) [47].

All the analyses were implemented in R software (version 4.1.3 https://www.r-project.org/). The IRT models were estimated using the mirt R package with the 'Rasch' option. The clustering was applied with the HCPC function of the FactoMineR package [48] and the interpretation of the indices was made with the catdes (for categorical variable such as country) or condes (for numeric variable such as the number of pesticides) functions of the FactoMineR package. Principal Component Analyses were performed with the PCA function of the FactoMineR package.

3. Results

3.1 Indices: IRT results and interpretation

3.1.1 Detailed interpretation of indices related to beebread collected in *A. mellifera* colonies in apple orchards

As a proof of principle, we chose to interpret in detail the index of site characterisation for a single dataset: the pesticide residues detected in beebread collected from *A. mellifera*

281 colonies in the 62 apple orchard sites (Table 2). The complete set of the indices' values for
282 each site and the interpretation of the indices are given in Tables A.1 to A.4 (in supplementary
283 material).

284 According to their index values, the sites were separated into four clusters. The statistical
285 differences between clusters highlighted the unequal repartition of detected pesticides. In
286 other words, if a pesticide was detected (respectively not detected) in a limited number of
287 clusters, it was qualified as an over-represented (respectively under–represented) pesticide.
288 If a pesticide was present in all the clusters, it was not considered as over-represented.
289 Pesticides were less present in Cluster 1 (N=10 sites out of the 62) than the mean calculated
290 across all sites. It presented the lowest index value (-1.32). Only a few pesticides (mean of
291 3.90) were detected in samples and none were over-represented compared to the mean.
292 Estonian sites were the most frequent in this cluster. Cluster 2 (N=12) did not contain sites
293 over or under-represented compared to the mean. The index value was negative (-0.49) but
294 higher than cluster 1's, meaning than cluster 2's sites were exposed to fewer pesticides than
295 the mean calculated across all sites but exposed to a higher number of pesticides than the
296 sites in the cluster 1. Cluster 3 (index value of 0.16) contained most of the sites (N=21) though
297 no pesticide nor country was over- or under- represented. Cluster 4 (N=19, index value of 0.83)
298 included the sites exposed to a high number of pesticides with 30 pesticides over-represented
299 compared to the mean. One insecticide (flonicamid) and five herbicides were the most
300 significant pesticides ($p < 0.005$). The concentrations ranged from 9 230 for the dithianon to
301 78.2 µg/kg for the flonicamid. The United Kingdom and German sites were over-represented
302 in this cluster and therefore hosted sites with higher number of detected pesticides. Swiss,
303 Irish and Swedish sites were significantly absent from Cluster 4. They were present in Clusters
304 1, 2 and 3 but not over-represented in any of these clusters.

Table 2 – Field site characterisation based on the index calculated on pesticide residues detected in **beebread** collected in ***A. mellifera*** colonies in the 62 **apple orchards** sites. CHE: Swiss sites. EST: Estonian sites. GER: German sites. IRL: Irish sites. SWE: Swedish sites. UK: The United Kingdom sites.

3.1.2 Overall description of the indices

All 18 indices were highly positively correlated with the number of pesticides detected in the matrices (mean correlation = 0.99; Table A.5, in supplementary material). This meant the higher the value of an index, the more exposed to a high number of pesticides the site was (details in Tables A.3 and A.4). Generally, matrices collected from apple orchards were exposed to a higher number of pesticides than matrices collected from oilseed rape crops, with respectively 7.6 [3.3-11.9] versus 3.5 [0.9-6.1] pesticides on average (details in Tables A.3 and A.4). Fungicides were highly present in the pesticides significant for the discrimination of clusters: 70% and 43.4% in apple orchards sites and in oilseed rape crops, respectively (Table A.6). Insecticides (20% and 33.9%, respectively) and herbicides (10% and 16.9%, respectively) were the other pesticide families the most represented. The quantities of these pesticides ranged from a minimum of 1.04 (insecticides) to a maximum of 9 230 µg/kg (fungicides) in apple orchard sites; and from 0.47 (for insecticides and herbicides) to 2 880 µg/kg (fungicides) in oilseed rape crop sites. Irrespective of the crop, pollen-nectar stores/beebread and pollen matrices contained a higher number of pesticides than nectar and forager matrices (Tables A.7 and A.8, in supplementary material). For apple orchards for instance, 15.1 and 10.4 pesticides were found respectively in beebread collected from *Apis* foragers and pollen from flowers whereas only 2.2 and 1.3 were found in nectar regurgitated from *Apis* foragers and in *Apis* foragers respectively. For oilseed rape, 14.9 and 7.7 pesticides were found in pollen-

329 nectar stores from *Bombus* foragers and pollen from flowers respectively, whereas only 1.2
330 were found in nectar regurgitated from *Bombus* foragers and 0.4 in *Bombus* foragers
331 themselves. It is worth noting that only 85 pesticides were screened for in nectar whereas
332 hundreds were screened in pollen-nectar stores/beebread, pollen and foragers. However,
333 despite the high number of pesticides screened for in foragers, only a few were found.

334 The pesticide residue presence in **pollen-nectar stores/beebread** collected from bees in apple
335 orchards was high in sites located in Italy for *Bombus* and *Osmia* species and in Germany and
336 the United Kingdom for *Apis* species. It was low in Estonian sites, irrespective of bee species
337 (Figure 3, Table A.3 and A.7). When looking at the pesticide residue presence in pollen-nectar
338 stores/beebread collected from bees in oilseed rape, the least exposed sites were in Estonia
339 for *Apis* and *Osmia* species and in Switzerland for *Bombus* species (Figure 3 and Table A.4). In
340 addition, sites located in Germany and Spain for *Apis* species and in Italy for *Osmia* species
341 were the most exposed according to the indices for pollen-nectar stores/beebread. No
342 country was over-represented in the exposed oilseed rape sites for *Bombus* species. Pesticides
343 that characterised the indices were different between the two crops. For a given crop,
344 different pesticides characterised the indices related to pollen-nectar stores/beebread from
345 the different bee species. In other words, pollen-nectar stores/beebread collected by the
346 three species did not contain the same type of pesticides irrespective of whether sampling
347 sites were in apple orchards or in oilseed rape crops. However, the characterisation of the
348 sites with a higher number of pesticides surrounded by oilseed rape included DMF (one
349 metabolite of the acaricide amitraz) for pollen-nectar stores/beebread collected from *Apis*
350 (3.49 µg/kg) and *Bombus* species (7.9 µg/kg) and the herbicide S-metolachlor for pollen-nectar
351 stores/beebread collected from *Apis* (3.93 µg/kg) and *Osmia* species (122.1 µg/kg).

352 Irrespective of the focal crop, the pesticide residue presence in **pollen collected from flowers**
353 was low in Spanish sites (Figure 3, Tables A.3 and A.4). The insecticide diflubenzuron (17.7 and
354 80 µg/kg, respectively) and the fungicide dimetomorph (15.6 and 58.3 µg/kg, respectively)
355 characterised the sites with a higher number of pesticides for pollen collected from apple
356 orchard and oilseed rape flowers. (Tables A.3 and A.4).

357 Looking at **pollen loads** collected from honeybee colonies in apple orchards, pesticide residue
358 presence was high in sites located in Germany and low in sites located in Spain (Figure 3 and
359 Table A.3). For honeybee pollen loads collected in oilseed rape sites, no sites were over-
360 represented in the highest cluster but Italian sites were over-represented in the lowest cluster
361 (Figure 3 and Table A.4). Different pesticides characterised the indices related to pollen loads
362 in the two crops. In other words, pollen loads collected from honeybee colonies did not
363 contain the same type of pesticides in apple orchards or in oilseed rape crops.

364 According to the indices, the **nectar** samples contained a higher number of pesticides when
365 collected in the United Kingdom sites in apple orchard, and fewer pesticides in Italian sites in
366 oilseed rape irrespective of the bee species (Figure 3, Tables A.3 and A.4). The characterisation
367 of the sites with a higher number of pesticides in apple orchards included the fungicide
368 epoxyconazole (2.43 µg/kg in nectar collected by honeybees). It was also present in nectar
369 (2.7 µg/kg) regurgitated from bumblebees collected in by oilseed rape sites and characterised
370 the sites with a higher number of pesticides.

371 When looking at pesticides present in **bees** collected from apple orchards sites, the indices
372 indicated that sites located in the United Kingdom had the highest number of pesticides and
373 those located in Estonia had the lowest, irrespective of the bee species (Figure 3, Tables A.3
374 and A.4). The pesticide residue presence in bees in oilseed rape crops was low in Irish sites for

375 *Apis* species and in Spanish sites for *Bombus* species (Figure 3, Tables A.3, A.4, A.7 and A.8).
376 No country was over-represented with respect to oilseed rape in the most exposed (in terms
377 of number of detected pesticides) sites. The characterisation of the most exposed sites in
378 apple orchards included the pesticide 1,2,3,6 tetrahydrophthalimide (metabolite of a foliar
379 fungicide Captan) for bees collected from both species (700.2 µg/kg in honeybees and 2 170
380 µg/kg in bumblebees). It was also present in bumblebees collected in the most exposed sites
381 in oilseed rape crops (197 µg/kg). The insecticide tau-fluvalinate characterised the most
382 exposed sites in oilseed rape crops independently of the bee species. The fungicide boscalid
383 characterised the most exposed sites in both crops for bees collected from *Apis* species (176
384 µg/kg in apple site and 275.2 µg/kg in oilseed rape sites).

385 For indices related to the matrices collected in apple orchards, the clusters of sites with the
386 highest rank of exposure included sites from either Germany, Italy or the United Kingdom
387 (Figure 2). The clusters with the lowest rank of exposure included sites from either Estonia or
388 Spain. Irish and Swiss sites were never over-represented in clusters for these indices. For the
389 indices related to the matrices from sites in by oilseed rape crops, the clusters of sites with
390 the highest rank of exposure included sites from either Germany, Italy or Spain. The clusters
391 with the lowest rank of exposure included sites from either Estonia, Ireland, Italy, Spain or
392 Switzerland. The United Kingdom and Swedish sites were never over-represented in clusters
393 for these indices.
394
395 Figure 2 – Summary of the sites that were most over-represented compared to the mean (p-value <0.05) in the
396 clusters with low (yellow) and high (blue) number of pesticides based on IRT index values for the nine matrices.
397 Sites in apple orchards are at the top of the figure, whereas those in oilseed rape are below. The bars mean that
398 no sites were over-represented compared to the mean in a cluster.
399

3.2 Links between the indices

The links between indices were illustrated by means of a PCA for matrices collected in apple orchards and in oilseed rape crops (Figure 3). The PCA correlation circles of variables (left plots) represented the link between the nine indices related to each matrix for a given crop. The plots on the right represent the 64 sites, the country being considered as a supplementary information. In data from apple orchard sites, 74.8% of the overall inertia was explained. Inertia is the overall information contained in the data. The remaining 15.6% of missing values were imputed. In data from oilseed rape sites, 51.3% of the overall inertia was explained. The remaining 10.8% of missing values were imputed.

Irrespective of the crop (Figure 3), the positive correlations between the nine indices meant that the number of pesticides measured in the various matrices varied in the same way. As indices and number of pesticides were highly correlated (section 3.2.2), the more detected pesticides there were in any given matrix, the more there were in related matrices. However detected pesticides were hardly the same.

Figure 3 – Graphical display of the first two components of the Principal Component Analysis of the nine indices (left) from the 64 sites (right) in **apple orchards** (A) or **oilseed rape crops** (B), the country being considered as a supplementary information. The interpretation arrows indicate the nature of the matrices regarding their content of fat (lipophilic, they attract molecules that dissolve in fats) and water (hydrophilic, they attract molecules soluble in water – see discussion for details) and their level pesticide content (low or high number of pesticides – details are given in the text).

In the apple orchard sites (Figure 3A left), two bundles of variables were highlighted: on one hand, indices related to nectar regurgitated from *Apis* and *Bombus* foragers and to *Apis* and *Bombus* foragers themselves, and on the other hand, indices related to pollen-nectar

424 stores/beebread collected from colonies and nests, pollen collected from flowers and pollen
425 loads from *Apis* traps. The indices related to nectar were highly correlated with each other
426 (cor=0.69) as well as with bumblebees (cor=0.47 for *Nectar.Apis*/*Bombus* and cor=0.60 for
427 *Nectar.Bombus*/*Bombus*). The indices related to pollen-nectar stores/beebread collected in
428 honeybee or in bumblebee colonies were highly correlated with each other (cor=0.83) and, to
429 a lesser extent, to the one collected in solitary bee nests (cor=0.79 for *Pollen-nectar*
430 *stores.Osmia*/*Beebread.Apis* and cor=0.83 for *Pollen-nectar stores.Osmia*/*Pollen-nectar*
431 *stores.Bombus*). These three indices related to pollen-nectar stores/beebread were also linked
432 with the pollen collected from flowers (cor=0.72 to 0.75) and with the pollen loads collected
433 from *Apis* traps (cor=0.65 to 0.72).

434 Some Italian apple orchard sites were the most exposed for pollen collected from flowers and
435 from *Apis* traps, pollen-nectar stores/beebread collected in colonies and nests from the three
436 bee species and honeybee foragers, whereas some the United Kingdom sites were the most
437 exposed for nectar regurgitated from both bee species and bumblebee foragers (Figure 3A
438 right). In Estonian, Spanish and Swedish sites, pesticide were less found in the matrices in
439 general. In some countries (Ireland, Italy and Sweden), the levels of exposure were highly
440 variable, whereas in others (Estonia, Spain) the levels were homogeneous.

441 In the oilseed rape sites (Figure 3B left), three bundles of variables were highlighted: (i) indices
442 related to pollen-nectar stores/beebread and pollen from flowers, (ii) indices related to *Apis*
443 and *Bombus* foragers, and (iii) indices related to nectar regurgitated from foragers and pollen
444 from *Apis* traps. The indices were less correlated than indices from the apple orchard sites. In
445 the oilseed rape sites, the indices related to nectar were correlated with each other (cor=0.63
446 for *Nectar.Apis* and *Nectar.Bombus*). The indices related to pollen-nectar stores/beebread

(*Beebread.Apis*, *Pollen-nectar stores.Bombus* and *Pollen-nectar stores.Osmia*) were moderately correlated with each other (cor=0.31 to 0.45). These three indices related to pollen-nectar stores/beebread were also slightly correlated to the pollen collected from flowers (cor=0.11 with *Beebread.Apis*, cor=0.23 with *Pollen-nectar stores.Bombus* and cor=0.41 with *Pollen-nectar stores.Osmia*).

Italian sites, and to a lesser extent, the German, Spanish and Swiss sites contained the highest number of pesticides for pollen from flowers and pollen-nectar stores/beebread. In Estonian and Irish sites the matrices contained the lowest number of pesticides in general (Figure 3B right). In some countries (Germany and Sweden) the number of detected pesticides was highly variable whereas in some others (Italy and Spain), it was rather homogeneous.

4. Discussion and conclusions

While several surveys have explored the presence of pesticides at the same time in different matrices [19, 34, 49], none proposed an index to characterise the exposure to pesticides. In this paper, we presented a highly novel statistical method using the IRT models to summarise complex information on pesticide presence into a single, yet interpretable, index.

4.1 Indices from IRT models: strengths, adaptation and limits

This index illustrated the exposure to pesticides. It was more informative than a classic assessment of richness or abundance because it took into account the overall repartition of pesticides between samples together with quantities of pesticides. This index made possible the calculation of clusters based on similarity or dissimilarity of samples in terms of pesticide

469 detection. As a consequence, comparison between sites (based on pesticide detection in the
470 different samples collected in a given site) was possible.

471 Before choosing IRT models, different statistical methods were considered to reduce the
472 complexity of the 18 datasets that originated from bee exposure to apple orchards and oilseed
473 rape crops including the Multiple Correspondence Analysis (MCA) [50] applied on the overall
474 distance matrix [51]. Contrary to the indices summarising the exposure to infectious and
475 parasitic agents (IPAs) [52], the MCA was not adapted to deal with the multidimensionality of
476 our data, as there was a very slow decay of eigenvalues due to the strong association between
477 sites and pesticides. The proposed indices revealed a structure related to the number of
478 pesticides detected on the sites, illustrated by the linear link between the number of
479 pesticides detected and the exposure level of the sites (the index). The clustering of the sites
480 based on the indices showed a clear separation between the clusters (Tables A.3 and A.4).

481

482 **4.2 Links between matrices and species**

483 When designing the site network, one goal was to explore land-use management across
484 countries and across agroecosystems, resulting in a gradient of exposure to pesticides [40].
485 The land-use management data will be used in forthcoming statistical analyses. Eight countries
486 from four biogeographic zones and two crops were included in the site network. The country
487 of origin was not considered for the index calculation. However, this additional information
488 was very useful to explain the different exposure levels at the sites. Applied to our dataset,
489 the indices showed that in general, matrices collected in apple orchards contained a higher
490 number of pesticides than matrices collected in oilseed rape crops. For a given matrix and a
491 given country, different pesticides characterised the exposure at the sites according to crop

exposure. These differences resulted from the crop treatments that were also different from country to country, most probably because of weather constraints and the blooming stage when sampling was performed. However, other factors may explain the diversity of pesticide uses across European countries such as the type of soils, the cultural habits and the commercial strategies from the pesticide industry.

In all cases, further statistical analysis is needed to compare the pesticide residue results to the real use of pesticides in the different countries. In other words, it would be worth investigating if, in the example of bees, the 1,2,3,6 tetrahydrophthalimide was more applied on apple orchards in the United Kingdom sites than in Estonian sites. Statistical analysis could focus on field treatments recorded during PoshBee; and on the theoretical number of formulations with a market authorisation in these countries. To our knowledge, such comparison has never been made.

In general, the same countries had the most exposed (Germany and Italy) or the least exposed sites (Estonia, Spain) irrespective of the analysed matrix and the crop. However, there was some variation in pesticide detection between matrices for example between beebread collected in *Apis* bees and nectar regurgitated from *Apis* bees in oilseed rape sites located in Italy and Spain. These results show the difference of use and application of pesticides between European countries. This could be further explored with analyses including additional data on pesticide availability in the European countries. Our results also give first insights in the pathway of the contamination chain to understand the source and effect of pesticide residues on bees as aimed at by the site network [40]. For a given site, all matrices contained similar number of pesticides but not necessarily by the same pesticides.

514 At apple orchard sites, the PCA highlighted the discrimination between pollen-nectar
515 stores/beebread and pollen indices from nectar and bee indices. This separation was expected
516 due to the high fat content of pollen-nectar stores/beebread and pollen and the high water
517 content of nectar. This matrix discrimination was independent of country. To our surprise, the
518 indices from the bee matrices (honeybees and bumblebees) were associated with the
519 hydrophilic matrix (regurgitated nectars) rather than lipophilic matrix. It should be noted that
520 this discrimination is based on pesticide numbers, as mentioned before. To further understand
521 the matrix partition, it would be worth looking at the type of pesticides found in the sites, and
522 checking if their chemical characteristics (lipophilicity, use of pKa) are in accordance with the
523 discrimination of the matrices.

524 Consistently across bee species, sites were exposed at the same level for a given matrix. Some
525 pesticides were in common, but in general the detected pesticides were different between
526 the bee species. The three focal bee species selected in this study differ in foraging distances
527 from <1 km for solitary bees [53] up to 6 km for honeybees [54] and foraging preferences.
528 Thus, they probably foraged to different extents on the two focal crops, other flowering crops
529 and wild plants, contributing to different detected pesticide exposure levels. This question will
530 be further explored with the palynological data analysis of pollen-nectar stores/beebread and
531 published in future papers.

532 The number of samples collected from *Osmia* bees were either reduced (for the pollen-nectar
533 stores) or absent (for the regurgitated nectar and for the bee bodies). This was an unfortunate
534 side-effect of the ecology and biology of this species. If the difficulty to retrieve this matrix
535 could be overcome, it would be worth examining the characteristics of pesticides (family,

active ingredients and quantities) found in *Osmia* pollen-nectar stores compared to the ones found in pollen-nectar stores/beebread from the other two bee species.

Although there was a tendency for the UK, German, and Italian sites to be the most exposed and the Spanish and Estonian sites the least exposed, there were exceptions according to matrices. For example, sites located in Italy were the least exposed when looking at the pesticide residue presence in nectar regurgitated from *Apis* and *Bombus* foragers and pollen loads collected from *Apis* traps following oilseed rape exposure (Tables A.1 to A.4).

4.3 Chemicals analysis as a key point to compare results on pesticide detection

The four laboratories involved in the analyses used different methods with large variation of screened pesticides depending on the extraction procedures and the analytical devices used [31, 55]. Ring tests between the different analytical laboratories could be implemented to produce comparable results. This preliminary work should be taken into consideration in future surveys. Usually, stock standard solutions are used to calibrate the analytical devices, with ready-to-use solutions containing several active ingredients. The non-availability of these stock standard solutions depending on the countries was a key point, preventing from having a common list of active ingredients screened for across the four laboratories. However, the list of 64 common pesticides to be screened in all the matrices defined before analyses enabled statistical comparisons when looking at analytical results. Many pesticides were included in the lists of screened pesticides and of those relatively few were found in the matrices – maximum 37% in beebread collected from honeybee colonies (Table 1). These results show that more reflection should be made on targeting analyses to reduce the number

558 of screened pesticides without impairing analytical relevancy. Indeed chemical analyses have
559 potentially important economic and ecological costs.

560

561 **4.4 Risk posed by pesticide residue presence in various matrices**

562 The IRT-based indices focused on bee exposure, not on risk assessment. However, considering
563 the toxicity of detected pesticides is key for the assessment of pesticide risks for different bee
564 species [56] and is linked to the quantities of pesticides in the different matrices. The
565 pesticides significant for discrimination (Table A.6) were mainly fungicides (70% in matrices
566 collected in apple orchard sites, and 43.4% in those surrounded by apple). The proportion was
567 the other way around for insecticides, more frequently found in apple orchard sites compared
568 to oilseed rape. Being more toxic to bees, the exposure to insecticides puts bees more at risk
569 than fungicide exposure. However, quantities and exposure scenarios are also important and
570 should be integrated in the calculation of risk indicators. It would be interesting to explore
571 whether the sites would be similarly clustered for pesticide risk, e.g., assessment based on
572 hazard quotients [34, 49, 57, 58] as regards to exposure, and if correlation between matrices
573 would be similar. In other words, would the risk posed by pollen-nectar stores consumption
574 to bumblebees be positively correlated to the risk posed by beebread consumption to
575 honeybees? Such statistical work should be further explored. Another way to look at these
576 data would be to explore the correlation between the cumulative concentrations of pesticides
577 and the IRT-based indices for each site. If there was a correlation, we could discuss the notion
578 of toxicity. It would be very interesting to have a comparison between cumulative
579 concentrations and added toxic units such as toxicity-weighted concentration [59, 60].

Future studies could further assess whether pesticide residue exposure was related to bee population traits recorded in the field [40] along with further potential stressors of bee health [61]. In a previous study, we proposed an index calculation to summarise the exposure to IPAs [52]. The two kinds of indices (IPA and pesticide exposure) could be related to each other or used in structural modeling equations to understand the drivers of bee health. PoshBee data from the site network made it possible to assess pollinator development under field conditions, which is likely more informative for real world scenarios than tests conducted in laboratory conditions [62]. Comparing the pesticides found in the different matrices is also of importance and should be conducted in future statistical works.

To conclude, the index calculation based on the IRT methodology presented in this paper is reliable and offers many applications. The characterisation of sampling sites based on the number of detected pesticides across different matrices enabled us to summarise information from complex samples into a single and interpretable index. Our results show that although pesticide numbers were similar in matrices from any given country irrespective of bee species, some important variations could be observed. Therefore, for a complete assessment of pollinator pesticide exposure, it is necessary to consider several different exposure routes and multiple species of bees across different agricultural systems. Other parameters should be considered such as bee population traits, different pesticide and application use between countries, other potential stressors of bee health. However all these information are usually lacking in field studies.

These results highlight the variation in the use and application of pesticides across European countries. This could be further explored with analyses including additional data on pesticide availability in the European countries. Our results also give first insights in the pathway of the

603 contamination chain to understand the source and effect of pesticide residues on bees as
604 aimed at by the site network [40]. For a given site, all matrices experienced similar number of
605 pesticides but not by the same pesticides or in comparable quantities.

606 Beyond such summarisation of complex data, the indices can be used in many ways, e.g. to
607 compare and explore the correlation between matrices. Our datasets and matrices offer
608 important opportunities for statistical analyses to examine relationships of the presented IRT
609 indices with risks posed by pesticides to pollinators or their influence on bee health.

610

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625

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