Agrochemical Contamination of Honey and Bee Bread Collected in the Piedmont Region, Italy

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Abstract: This study shows the results of a local biomonitoring plan developed by a regional beekeeping association, Aspromiele, in several areas of Piedmont (Italy), in order to understand the status of contamination from pesticides present in the environment and eventually to evaluate their impact on apiculture. Glyphosate was the most abundant chemical found in the bee bread and honey samples. The other pesticides detected at lower concentrations and minor frequency were mandipropamid, tau-fluvalinate, metalaxil and spiroxamine. Even if in the present study the pesticides found in the bee bread and honey were limited to a few molecules, it is important to highlight that the presence of glyphosate could represent a hazard to bees. Honeybees are the main pollinators in agricultural ecosystems, and thus appropriate environmental management could lead to a reduction in the impact of these chemicals on bees and other beneficial insects.

Keywords: honey; pollen; pesticides; Italy

1. Introduction

Honeybees (Apis mellifera) are extremely vulnerable to pesticide contamination as they are exposed to these substances while exploring the environment surrounding the hive and while collecting pollen, nectar and water from available sources [1]. The protection given by the most commonly utilized pesticides often leads to the contamination of all plant organs, including flowers. The final receiver of these contaminants are then nectar and pollen. While nectar represents the carbohydrate input for the bee colonies, fundamental to the foraging bees and the surviving of the families, pollen is the main protein and lipid source and a part of the nurse bee and larval diet [2]. For that reason, the contamination of these floral parts result in exposure of all the stages present in the bee colonies: the new generation, the foraging and the receiver bees [3]. Unfortunately, widespread pollen contamination from agricultural landscapes often have been reported [4–9]. Furthermore, significant correlations were found between the presence of fungicide residues and honeybee colony disorders and between the latter and the abundance of crop surface around the apiaries [10], the proximity of the contamination source and the duration of exposure [11–14]. Bee pollen can be used as an indicator of environmental contamination [1], as sorption studies indicated that the pesticides could bound in this matrix. Furthermore, pollen is easy to collect and is largely contaminated [4,12]. Among the bee products, beeswax, honey and pollen have been verified to represent an appropriate sentinel for monitoring environmental contamination for persistent organic pollutants and pesticides [15–17]. The palynological spectrum can thus reflect the flora collected by bees during the active season [18].

Since in agricultural practices the use of several herbicides, insecticides and fungicides is allowed for plant protection, bees can be exposed to cocktails of chemical compounds
that can affect not only bee individuals but also colony viability. While it is reported in
several studies that high levels of insecticides in the short term can produce mortality
in honeybees [17,19], the most recent studies have shown that lower, sublethal doses
can impair the bees’ behavior [20,21], learning [22–24], colony development [25,26], and
can cause immune and nutritional stress, increasing the susceptibility to varroa or other
pathogens [27–29]. Unfortunately, widespread pollen contamination from agricultural
landscapes often have been reported [4–9].

The effects of the chemicals on the bees could depend to the chemical class. Pyrethroids,
for example, have reported repellant effects on foragers, and exposure causes already-
foraging bees to decrease foraging activity [22] and increases the number of non-foraging
behaviors exhibited by these foragers [30], while several fungicides can negatively af-
fect honeybees in a way that resembles nutrition deficiencies or weakens honeybees by
promising the immune system, thus increasing susceptibility to parasites. Furthermore,
significant correlations were found between the presence of fungicide residues and
honeybee colony disorders and between the latter and the abundance of crop surface
around the apiaries [10], the proximity of the contamination source and the duration of
exposure [11–14].

Neonicotinoids, that were recently restricted for outdoor use in the EU [31], are ag-
onists of the nicotinic acetylcholine receptors (nAChR), mainly circulated in the insect
central nervous system, and can interrupt processes related to cholinergic neurotransmis-
sion, such as olfaction, learning, and memory [32]. They might affect the foraging behavior
or induce motor impairments, thereby limiting foraging bees and having the most serious
consequences for colony performance [33].

Finally, we need to consider the wide use of herbicides, looking with particular interest
at glyphosate that represents the best-selling pesticide in the world, accounting for 71.6%
of the active ingredients marketed [34]. Several studies consider glyphosate as a product
practically non-toxic or slightly toxic to animals [35–37], while others reported toxic effects
also on honeybees and other pollinators [38–40]. It has been reported that it can have
negative effects on a bee’s behavior, influencing their capacities to return successfully back
to the hive [41], but also decreases their food resources, reducing the diversity of plants
around the crop and, consequently, reducing pollen and nectar [42].

Another important factor that might influence the toxicity is related to the common
practice of mixing pesticides. For instance, mixing insecticides with certain fungicides
can synergize the acute toxicity of the insecticides to honeybees. The activity of some
pyrethroids is enhanced by fungicides classified as ergosterol biosynthesis inhibitors while
the fungicide propiconazole increases the toxicity of the pyrethroid insecticide, lambda-
cyhalothrin, when the two are mixed [42].

Considering the honeybees as the primary pollinator in agricultural landscapes, this
study is a limited survey but important to understand the magnitude of pesticide use in a
selected area of Piedmont, Italy. Here, the regional beekeeping association, Aspromiele,
has developed a monitoring plan in order to understand the environmental contamination
from pesticides. This study shows only the results of a short monitoring period of 2 years
(2019/2020), and it analyzes two important hive matrices, fresh honey from the nest comb
and bee bread. The experimental apiaries were located not only near agricultural settings,
but also in organic farms and mountain areas. The pesticide presence was evaluated
periodically in the hive matrices, in association with palynological analyses useful to
understand the origin of the chemical pollution.

2. Materials and Methods

2.1. Honey and Beebread Sampling

Four apiaries located in Piedmont (Italy) were included in the monitoring, each
of them presenting two observed colonies. Two apiaries were located in proximity of
agricultural areas (number 1 and 3); one (number 4) in the mountain (850 m a.s.l.), out of
any intensive agricultural context; and one (number 2) in the middle of organic hazelnut
farms (Figure 1). The colonies were managed by beekeepers according to the organic production protocols [43].

![Map of Monitored Apiaries in Piedmont, Italy](image)

**Figure 1.** Monitored apiaries in Piedmont, Italy.

The honeybee families consisted of healthy and queen-right colonies presenting no other diseases than a low level of varroatoasis and they were bred in Dadant-Blatt hives, on 10 combs. The field trial started in March 2019 and finished in September 2020. Both years the colonies were monitored between March and September for a total of 7 observations and samplings a year. Each month, samples of the fresh honey and bee bread were collected from the monitored colonies. The population of the families were determined by the ColEval method, from May to September 2020. The ColEval monitoring tool is based on the evaluation of the surface area percentage occupied by the components of a honeybee colony (adult worker bees, open and capped brood, honey and pollen). The percentage evaluation
was transformed into numbers or surface areas thanks to the coefficient transformation values calculated by Hernandez et al. [44].

Two different matrices were sampled from each station: bee bread and fresh honey. Both these matrices were collected from the nest combs. Each sample, both for honey and bee bread, consisted of the excision of 5 cm × 5 cm of the comb.

Furthermore, in case of abnormal mortality of the honeybees, the presence of pesticides in the environment was also monitored using the underbasket cages to collect dead bees, outside the hives. Environmental data close to the hives were monitored using a thermometer and a hygrometer, located 40 cm from the ground.

The main crops in and around the experimental station number 1 and 3 were polyphite meadows; cultivated fields of wheat, corn and barley; hazelnuts; and spontaneous vegetation. Sampling station number 4 was in the middle of native forest and spontaneous polyphite meadows while the area surrounding sampling station number 3 was cultivated by hazelnuts.

Each sample was transported to the laboratory in a plastic container, then stored at −20 °C until the analysis.

2.2. Palynological Analyses

The palynological analysis was carried out according to von der Ohe et al. [45], properly adapted for pollen analysis. Two grams of pollen for each sample were dispersed in 50 cm³ of distilled water and an aliquot of 0.01 cm³ of this suspension was fixed onto a microscopic slide. At least 1000 grains for each slide were counted. The raw palynological spectra, defined according to the nomenclature proposed by Persano Oddo and Ricciardelli d’Albore [46], were then converted to the volumetric ones to reflect real pollen mass instead of grain counts [47]. For this purpose, a database of the average pollen grain’s volume was produced, based on the grain’s dimensions reported by the Ponet database (AGES, 2016) and according to the procedure proposed in Conti et al. [48].

2.3. Multiresidue Pesticide Analysis of the Bee Bread and Honey

Samples were analyzed for 67 chemicals belonging to the herbicide, insecticide, acaricide and fungicide toxicological classes (Table 1). The extraction and purification method for the pollen have been described in a recently published work [3].

Table 1. Chemical compounds searched by GC-MS/MS and LC-MS/MS in pollen and honey.

| Searched Molecules | Acaricides | Fenazaquin, Propargite, Tebufenpirad |
|--------------------|------------|--------------------------------------|
| Fungicides         | Linuron, Propyzamide, Glyphosate        |
|                    | Azoxystrobin, Benalaxyl, Biteranol, Boscalid, Cyzofamid, Dichlofluanid, Difenconazole, Diethofencarb, Fenarimol, Fenbuconazole, Fenexamid, Fluopicolide, Flusilazole, Imazalil, Iprovalicarb, Kresoxim methyl, Mandipropamid, Metalaxyl, Nuarimol, Oxadixyl, Spiroxamine, Tebuconazole, Thiabendazole, Tolyfluanid, Trifloxystrobin, |
| Insecticide        | Difenoconazole, Dithofencarb, Fenarimol, Fenbuconazole, Fenexamid, |
|                    | Acrinathrin, Azinphos-methyl, Bifenthrin, Buprofezin, Carbaryl, Chlorfenvinphos, Chlorpyriphos-ethyl, Chlorpyriphos-methyl, Coumaphos, Chlothianidin, Cyfluthrin, Diazinon, Dichlorvos, Dimethoate, Esfenvalerate, Ethion, Etofenprox, Etrifmos, Fenitrothion, Fenthion, Fosmet, Heptenofos, Imidacloprid, Lambda cyhalothrin, Malathion, Mevinphos, Parathion ethyl, Parathon methyl, Phenthoate, Pirazophos, Pirimicarb, Pirimiphos ethyl, Pirimiphos methyl, Quinalphos, Tau-fluvalinate, Thiamethoxam, |
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Italy). Dispersive solid-phase extraction (dispersive-SPE) cleanup was done to remove any impurity, excess water, and other components with a combination of primary secondary amine (PSA) sorbent and MgSO$_4$; then the extracts were analyzed by LC-GC MS/MS (AB-SCIEX Instruments, Foster City, CA). The method for honey was optimized on the basis of UNI EN 15662 and AOC 2007.1, which is ISO 17025 accredited.

For the determination of glyphosate, each sample of honey/bee bread consisted of 5 g, mixed with 9 mL of ultra-pure water extracted in a single-step acidified methanol (10 mL acidified with 1% v/v formic acid) extraction. The extracts were analyzed by LC-MS/MS, as indicated in the QuPPe M 1.3 method (Quick Polar Pesticides Method) QuPPePO (Products of Plant Origin and Honey, EU Reference Laboratory for pesticides requiring Single Residue Methods (EURL-SRM)).

The limit of detection (LOD) was estimated using the method based on the signal-to-noise approach. A signal-to-noise ratio of 3:1 was considered acceptable for estimating the LOD. The LOD range was from 0.25 ng/g to 3 ng/g, while the limit of quantification (LOQ) from 2.50 ng/g to 10 ng/g for all pesticides except glyphosate, which reported an LOD of 3 ng/g and an LOQ of 10 ng/g.

2.4. Statistical Analysis

To compare contamination values in different years, the Wilcoxon signed-rank test was applied. SPSS® 14.0.2 (SPSS Inc., Chicago, IL, USA) was used as the statistical package.

3. Results

Results of the pesticides found in bee bread and honey are reported in Tables 2 and 3. A total of 66.6% of the 84 samples analyzed in the present investigation reported the presence of one or more chemicals. A significant difference ($p < 0.002$) was found for the values of glyphosate in the bee bread samples collected in 2019, showing a reduction in the year 2020. No significant differences were found for the honey samples.

### Table 2. Range, mean and standard deviation of the pesticides found in the honey samples coming from the different stations.

| Matrix | Sampling Site and Coordinates | Glyphosate (ng/g) | Mandipropamid (ng/g) | Metalaxil (ng/g) | Spiroxamine (ng/g) |
|--------|-------------------------------|-------------------|----------------------|-----------------|------------------|
| Honey  | 1(44.76297-8.104263)          | 10–34 (23 ± 11.40)| <LOQ-20              | <LOQ-4          | <LOQ-40          |
| Honey  | 2(45.04583-8.075938)          | 10–29 (17.5 ± 8.89)| <LOQ                 | <LOQ            | <LOQ             |
| Honey  | 3(44.59696-8.099426)          | 10–19 (13 ± 4.08)  | <LOQ                 | <LOQ            | <LOQ             |
| Honey  | 4(44.31935-7.259777)          | 10–16 (13 ± 4.24)  | <LOQ                 | <LOQ            | <LOQ             |

### Table 3. Range, mean and standard deviation of pesticides found in bee bread samples coming from the different stations.

| Matrix  | Sampling Site and Coordinates | Glyphosate (ng/g) | Tau-Fluvalinate (ng/g) | Mandipropamid (ng/g) |
|---------|-------------------------------|-------------------|-----------------------|----------------------|
| Bee bread | 1(44.76297-8.104263)           | 10–104 (37.75 ± 31.82) | <LOQ                 | <LOQ-10              |
| Bee bread | 2(45.04583-8.075938)           | 10–59 (24.70 ± 19.95)  | <LOQ-50              | <LOQ                 |
| Bee bread | 3(44.59696-8.099426)           | 20–542 (103.57 ± 193.98) | <LOQ                 | <LOQ                 |
| Bee bread | 4(44.31935-7.259777)           | <LOQ-49 (23 ± 22.52)  | <LOQ                 | <LOQ                 |
Among the 67 pesticides analyzed, only 5 were found in the bee bread and honey samples, but the highest frequency was associated with glyphosate (50% of samples). Comparing the two matrices analyzed in this study, the bee bread showed higher glyphosate mean values and these concentrations reflect the environmental contamination status [49].

As shown in Tables 1 and 2, glyphosate residues similarly contaminated the bee bread (53.6% > LOQ) and honey (50% > LOQ), showing no significant difference. No significant difference in contamination prevalence was also found when comparing the honey prevalence and the different sampling stations. For bee bread, sampling station number 3 reported a significant difference (p < 0.05) with respect to the other stations, but this result is due to the presence of an outlier in site number 3, where a sample reported a glyphosate concentration of 542 ng/g.

Results of the palynological analyses done on bee bread samples positive to the glyphosate are shown in Table 4.

### Table 4. Palynological results.

| Sampling Period | Sampling Site | Glyphosate (ng/g) | Pollen |
|-----------------|---------------|------------------|--------|
| March 2019      | 1             | 64               | Salix (91.9%) |
| March 2019      | 2             | 44               | Castanea (30.3%) |
| March 2019      | 3             | 46               | Prunus (52%) |
| April 2019      | 1             | 24               | Robinia (34.2%) |
| April 2019      | 2             | 56               | Quercus robur (24.7%) |
| April 2019      | 3             | 20               | Cruciferae (40.6%) |
| May 2019        | 2             | 11               | Trifolium repens (20.9%) |
| July 2019       | 3             | 542              | Graminaceae (16%), Compositae (28%), Plantago (16%) |
| August 2019     | 1             | 104              | Hedera (91%) |
| August 2019     | 2             | 59               | Hedera (93%) |
| August 2019     | 3             | 58               | Hedera (54%) |
| August 2019     | 4             | 49               | Hedera (87%) |
| March 2020      | 2             | 13               | Prunus (83.4%), Salix (6.2%) |
| March 2020      | 3             | 25               | Prunus (64.7%), Salix (16.7%) |
| March 2020      | 4             | 10               | Salix (86%), Prunus (12.8%) |
| April 2020      | 2             | 16               | Fraxinus ornus (70.9%), Prunus (7.3%) |
| April 2020      | 3             | 18               | Fraxinus ornus (55%), Aesculus (10.1%) |
| May 2020        | 1             | 10               | Papaver (64.3%), Amorpha (23%) |
| May 2020        | 2             | 14               | Trifolium repens (30.9%), Chamaerops (13.9%) |
| May 2020        | 3             | 16               | Papaver (34.3%), Castanea (28.6%) |
| June 2020       | 1             | 10               | Compositae (35%), Clematis (29.8%) |
| June 2020       | 2             | 12               | Rubus (76.2%), Trifolium repens (12.7%) |
| August 2020     | 2             | 12               | Castanea (78.8%), Rubus (9.3%) |

Results of the ColEval evaluation of the surface area percentage occupied by the components of the monitored honeybee colonies (adult worker bees, open and capped brood, honey and pollen) from each monitoring site, are presented in the Supplementary Materials.

### 4. Discussion

In the two years of monitoring, no abnormal mortality of honeybees in front of the beehive occurred, even if the underbasket cages have been present for such an occurrence during the whole monitoring period. Pollen, in the form of bee bread, is the honeybee’s main source of protein that are vital to brood production and to development of young bees. It also provides fats/lipids, minerals and vitamins. The presence of pesticides in bee bread or pollen have been reported worldwide [12–14]. All life stages in the colony are vulnerable to toxic exposure because chemicals can be integrated into the hive products or by air movements, and can be distributed into other compartments; also, it is important to consider that redistribution among the different compartments takes place according to the physico-chemical properties of the pesticide and the main characteristics of the hive matrices [50]. In these conditions, the chemical molecules present in beeswax or in other
hive matrices could be transferred to beebread or honey samples; thus, the compounds found in the hive could have two different origins directly from the polluted environment (primary exposure) or from hive matrices (secondary exposure). The sublethal effects of pesticides on bees could act by interfering with their physiological metabolism, immunity and tolerance to viruses and pathogens [51].

The mean glyphosate concentrations (17.1 ng/g) found in the present study for honey is in accord to results reported by Rubio et al. [52], where, in commercial honey from Spain, Greece and Hungary, the mean values were below the 20 ng/g level, while in the USA, the mean concentrations were higher. Probably this could be due to the greater use of this herbicide in other international countries with respect to Europe. Glyphosate residues were found in fifty percent (50%) of the analyzed samples, and also these data fit with that reported by Rubio et al. [52]. Furthermore, in this latter study, the presence of glyphosate was reported also in organic honey. In our study, glyphosate was detected also in bee bread from organic hazelnut farms (station number 2) during the 5-month sampling period from March to August. In fact, even three pollen samples and one honey sample coming from the station (number 4) furthest away from any intensive agriculture reported low concentrations of this herbicide. The limited data of El Agrebi et al. [53] suggest that, for glyphosate, the transfer from wax to honey do not occur and this could confirm the direct contamination of bees by the polluted environment. The highest glyphosate concentration found during the sampling period (July 2019, Table 4) was probably due to the use of this herbicide for managing stubbles after harvest.

The beebread and honey samples analyzed in the present study revealed the occurrence, even at very low concentrations, of glyphosate in each monitored area in Piedmont. The highest levels were found in bee bread from agricultural areas, but this is a compound very diffused in the environment, e.g., through flowers or draining rivers.

Considering the risk to human health, there are no regulations concerning pollen samples. Nonetheless, for honey, all positive samples reported a maximum glyphosate concentration of 34 ng/g, not exceeding the EU MRL set at 50 ng/g and thus theoretically posing no risk to the consumer.

5. Conclusions

Even if in the present study the pesticides found in the bee bread and honey were limited to a few molecules, it is important to highlight the presence of glyphosate, which could represent a hazard to bees. Glyphosate is indeed the most extensively used herbicide worldwide and its intensive use has led to the widespread contamination of different ecosystems [54]. Moreover, glyphosate and its primary metabolite, aminomethylphosphonate (AMPA), have been detected in immature seeds [55], harvested seeds and ground water [56]. Although this herbicide does not appear as toxic to bees as some other pesticides, glyphosate has been reported to perturb, at very low concentrations, the honeybees’ gut microbiota, changing the bees’ susceptibility to environmental stressors, including poor nutrition [57] and pathogens [58]. Furthermore, it could be considered that the pesticide risk for bees can increase when some class of these chemicals act synergistically, amplifying the adverse effects of non-chemical stressors. Further investigations are needed to assess the synergies with other pesticides and the effect of longer-term exposures at sub-lethal doses.

Supplementary Materials: The Supplementary Materials are available online at https://www.mdpi.com/article/10.3390/environments8070062/s1. Table S1. Numbers of adult bees for each monitoring site, Table S2. Numbers of capped brood cell for each monitoring site, Table S3. Numbers of open brood cell for each monitoring site, Table S4. Honey area for each monitoring site (dm²).

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