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Foraging Honeybees (*Apis mellifera ligustica*) as Biocenosis Monitors of Pollution in Areas Affected by Cement Industry Emissions

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Abstract: Two areas affected by cement plant emissions, in an industrial district of Central Italy, were investigated by foraging honeybees (*Apis mellifera ligustica*) on the return to their hives, as an in situ biomonitor. The contamination was compared with that of a background reference area on the Central Apennine Mountains, quite far from the contamination sources. At all the sites, the bee colonies were stationary. One hundred seventy-seven compounds belonging to the class of polycyclic aromatic compounds (PACs) were positively identified by gas chromatographic and mass spectrometric techniques. For the first time, the presence of several unusual compounds on bee samples is highlighted. These include polycyclic aromatic sulfur heterocycles (PASHs), 1.55–35.63 ng/g d.w., compounds that, like polycyclic aromatic hydrocarbons (PAHs), 67.50–129.95 ng d.w., are classified as carcinogenic and/or mutagenic. In an attempt to identify the contribution of different and specific sources of these pollutants to the total pollution profile, the composition of aliphatic linear hydrocarbons was also examined.

Keywords: honeybees; biomonitors; polyciclic aromatic compounds (PACs); polyciclcic aromatic hydrocarbons (PAHs); polycyclic aromatic sulfur heterocycles (PASHs); n-alkanes; environment pollution; cement industry

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1. Introduction

The first use of honeybee (*Apis mellifera*) as a potential bioindicator and bioaccumulator of the environmental quality status dates back to 1935, when the harmful effects of smelter effluents of Czechoslovak industrial areas on bee living and foraging areas would seem to be proven [1]. In the late 1950s, new scientific findings about bees and honey contamination by ^{90}Sr radionuclide from the fallout of atmospheric nuclear tests were found [2]. It was not unless 1961, for the first time, that systematic investigations were performed to detect pollution in honeybee colonies close to an industrial zone in Montana State (Washington, USA) [3]. As early as 1984, a review listed a considerable number of publications concerning bees as bioindicators of metals in the environment [4]. Since then, there has been a significant increase in interest in this regard. In fact, a lot of scientific evidence confirms the close relationship between this pollinator, its products (honey, propolis, pollen, and wax), and the habitat surrounding the hives, as well as the success key as a biomonitor [5]. Most of them concern its use as an 'ideal bioindicator' to monitor the degree of episodic or continuous contamination in the honeybees living and foraging area. The investigated pollution, due to toxic xenobiotics, such as heavy metals, Hg, Cr, Cd, Pb, etc. [6–14] and radioactive elements, ^{137,134}Cs, ^{131}I, ^{60}Co, ^{40}K, ^{7}Be, etc. [15–19], has been found in different contexts in all over the world: industrial, urban, rural and wildlife reserves. Only recently, a few studies have addressed the problematic aspects of residues of fertilizers, pesticides,

fungicides, and acaricides, including the new harmful neonicotinoid family [20–24], and even fewer and an even smaller number concerning Persistent Organic Pollutants (POPs), especially the ubiquitary Polycyclic Aromatic Hydrocarbons (PAHs) [25–28]. In addition, over the years, the numerical disparity between studies on inorganic and organic pollutants reflects the different degrees of development of analytical devices and the methodologies to characterize them. In fact, biological samples are extremely complex matrices to analyze. Until the relatively recent past, it was extremely arduous and laborious to treat organic xenobiotics both for the cleanup process and for chromatographic separations, as well as for the low sensitivity and specificity of the available instrumentations.

Notwithstanding that these pollinators are influenced by latitude, seasonality (spring and summer), and meteorological–climatic factors [29], their ecological, ethological, and morphological characteristics, as well as their unlimited availability and ease of management, make them efficient and global biomonitors of the area surrounding its living environment. Traditional stationary point control methods, such as active and passive air sampling, wet and dry atmospheric deposimeters, and water and soil collections restricted to a small area of interest and to a single environmental compartment at a time, could require several monitoring stations to investigate in time and space the distribution of pollutants over an area of some square kilometers. The bee performs all these tasks at the same time, continuously and systematically. This insect is characterized, in effect, by high mobility ranging over long distances, during their activities of collecting flower nectar and pollen in the ecosystem. Flying at 24 km/h, every day, they cover a large sector of ~7–10 km^2 by exploring an average radius of ~1.8 km around the hive [11,14,25]. In addition, individual honeybees make 3 to 10 foraging trips per day [12]. Each active pollinator flight is characterized by a few ten to a few thousand nectar micro-samplings needed to fill its honey stomach (crop) and pollen basket (corbicula). Consequently, the total flower collections from a healthy hive amounted to 1–300 million in a single day. These numbers are sufficiently representative to estimate simultaneously, as a *unicum*, the changes in all environmental compartments strongly interconnected to the honeybee's lived habitat: air, soil, water, flora, and fauna. Unfortunately, as reported rightly by Simon-Delso and co-workers [30], contaminants' nature and sources can not always be so immediately identified and traced. Therefore, in this regard, knowledge is sometimes lacking due to the little and incomplete data available. The difficulty of having a thorough and accurate knowledge of the origin and fate of individual pollutants is a result of their widespread dispersion, mobility from the emission source, and atmospheric leveling.

This is particularly true for those chemicals such as PAHs, among the most ubiquitous organic molecules in the world, having several different kinds: pyrolytic, petrogenic, and diagenetic [31]. In fact, the PAH fingerprints are characterized by both the incomplete pyrolysis of organic matter and the specific production activities from which they are generated.

As is well known, the areas of fallout emissions of the cement sector are heavily affected by primary pollutants such as sulfur dioxide, nitrogen oxide, and carbon monoxide (thousands of tons per year), as well as heavy metals and particular matter. In spite of this, emissions of compounds such as PACs and polychlorinated dibenzo-p-dioxins and furans should not be overlooked, as generated during clinker firing. In fact, the use of such plants as waste incinerators and the use of low-cost fuels during the production process is not so unusual, resulting in adverse effects on the environment and human health [32].

Therefore, the main objective of the present research is an attempt to assess contamination in local areas impacted primarily by cement plant emissions and to validate the effective honeybee abilities in pollution source identification. This was achieved by analyzing the foraging honeybee bodies for the residue levels of PAHs and, for the first time, for polycyclic aromatic sulfur heterocycles (PASHs). As a further complement, in order to exhaustively attribute each single emission source to the total pollution profile, the composition of linear aliphatic hydrocarbons was also investigated.

2. Materials and Methods

2.1. Sampling Sites

The biomonitoring study concerns two localities (sites 1 and 2) near an industrial district in central Italy (Umbria), impacted by cement plant emissions, Figure 1, over a period from May to August, time of full activity of honeybees. The area affected has an agricultural vocation.

Figure 1. Map of industrial district showing cement plants, affected sites (**1**, **2**), and pristine area (**3**) position and beehive locations.

One factory was located South Southeast and the second North Northwest of industrial area, about 8 km apart. During the investigation, both plants were powered by 'dirty' fossil fuels, coal, and one of them was used as an incinerator of scrap tires, carcasses, and skeletons as well. The apiaries were positioned just outside the areas affected by plant pollution, two little villages (site 1 and 2, ~500 m a.s.l.), and in pristine area (site 3, 1020 m a.s.l.) north of the emission sources on the Central Apennine Mountains, as "uncontaminated" background reference, sufficiently far from the sources (~10 and 18 km, respectively). Each apiary consisted of at least three bee hives [33].

2.2. Honeybee Samples

In the present study, the bee colonies were stationary at the monitoring sites for at least two years. To ensure homogeneity of samples compared to a collection of random bees, only foraging honeybees (*Apis mellifera ligustica*) on return to the hives were collected. In order to capture bees without the use of fumigator, avoiding the potential contamination of the samples to be analyzed, the front doors of the hives were screened with a mosquito net. After being caught alive with a glass jar, simulating what happens naturally in autumn and winter, the bees were cold-killed at 4 °C in refrigerator. All pools numbered no less than 150 foraging bees, 50 subjects per beehive, for each monitoring site. Samples were stored in food plastic containers and kept in the darkness at −20 °C until the chemical analysis. Honeybee drones and pollen present in bee hind legs were manually removed, then the bee pools were freeze-dried under vacuum (mod. Modulyo Edwards, England) for 5 days at −55 °C, quartered, and totally pounded in ceramic mortar. The assessment of the honeybee water content was evaluated by the percentage difference in weight of the representative pool and those arising from its freeze-drying treatment.

2.3. Analytical Procedures (Sample Extraction, Cleanup, and Analysis)

Residue-free analytical grade solvents (purity ≥99%; Sigma-Aldrich, Deisenhofen, Germany) were used for extraction and cleanup of honeybee samples. ISTISAN 99/28 standardized analytical method, slightly modified, was performed to ensure high quality assurance (QA) and quality control (QC) [34]. Recoveries of analytical procedure were

≥80%. Briefly, an aliquot of freeze-dried and pounded sample (1 g equivalent to ~27 insects) was spiked with a perdeuterated mix standards (namely naphthalene-d8, acenaphthene-d8, phenanthrene-d10, pyrene-d10, chrysene-d12 and perylene-d12) and then extracted by sonication twice with 10 mL of dichloromethane for 10 min. The two solutions were unified and reduced to small volume (~500 µL) under vacuum in a rotary evaporator. To clean up and to separate the analytical fractions, the extract residue was chromatographed on 3% *w*/*w* H_2O activated silica gel (~6 g Bondesil-SI, 40 µm—Varian) column. The first one, aliphatic hydrocarbons, was eluted with 10 mL of n-hexane, and the second one, PAHs and PASHs, was eluted with 20 mL of n-hexane-dichloromethane (4:1; *v*/*v*). The fractions were reduced to a few microliters (25–150 µL) under N_2 gentle flow. One µL of single fractions was analyzed by gas chromatographic techniques (GC) using a Varian-Chrompack 3800 GC coupled with a tandem mass spectrometry ion trap detector (ITD-MS; Varian Saturn 2000), equipped with a split–splitless inlet and a low bleed Factor Four VF-5 ms capillary column (Chrompack, Middelburg, The Netherlands), 30 m × 0.25 mm ID and 0.25 µm film thickness, with a 5 m of an uncoated fused silica pre-column as retention gap. For qualitative and quantitative characterization, the GC operative conditions were for aliphatic hydrocarbons: oven temperature from 90 °C to 300 °C at 15 °C/min. then to 300 °C for 15 min; injector operating in split mode (ratio 1:2) at 260 °C and for PAHs and PASHs: oven temperature from 90 °C to 180 °C at 10 °C/min., from 180 °C to 290 °C at 6 min. then to 290 °C for 18 min.; injector operating in splitless mode at 260 °C. Helium carrier gas flow was constant at 1.3 mL/min. The ITD-MS operative conditions were as follows: interface 240 °C, manifold 90 °C, and ion trap analyzer 160 °C. The EI+ mass spectra were obtained at 70 eV and 10 µA, in full scan acquisition for quantitative aliphatic hydrocarbons and qualitative PACs screening (range *m*/*z* 50–450), while in µSIS (Single Ion Storage) modality for quantitative PAHs and PASHs. For correct and unambiguous identification of each component of the analyzed fractions, experimental mass fragmentations were compared to standard mass spectra listed in NIST92 and Wiley5 libraries.

3. Results and Discussion

3.1. Water Content of Foraging Honeybee Samples

The bee water content of the samples analyzed was estimated after freeze-drying under the vacuum of a pool of eight bulks. The average value amounts to 62.0 ± 3.1%, confirming a uniform content of water in the different samples, regardless of either insect capture station or seasonality.

3.2. Non-Polar Aliphatic Fraction

The study of this homolog series, in particular of n-alkanes, provides useful knowledge on the nature of pollution with its unique and distinguishable distribution in all environmental compartments. Their constituents, in fact, can originate from both anthropic and biogenic sources. Congeners with chain lengths up to 20 carbon units (*n*C20) are generally connotative of the anthropic nature: vehicular emissions and combustion of charcoal and petroleum products; on the contrary, the highest carbon units originate primarily from biogenic, plant wax aerosols [35]. The difference can be usually recognized through the assessment of the bimodal distribution of these homologous and the diagnostic criteria as the Carbon Preference Index (CPI), empirical parameter estimating predominance of odd over even congeners (plant wax contribution vs. fossil fuel contamination), and carbon number maximum (C_{max}). All honeybee specimens analyzed, including those of background green reference area, showed a complex mixture of aliphatic compounds. Forty-four homologs were positively identified: n-alkanes, their mono-methyl branched derivatives, and mono-alkenes. The n-alkanes were the preponderant group. In Table 1, the concentrations, relative percent contributions, and carbon preference index CPI_{25} related to homolog 25 [36] of each monitoring sample are reported, while in Figure 2, comparative n-hydrocarbon distributions of individual homologs are reported.

Table 1. n-Alkane concentrations (expressed as ng/g honeybee d.w.), relative percent contributions (%), and Carbon Preference Index (CPI_{25}) of monitoring bee samples.

Homolog		Monitoring Site					
		1		2		3	
		ng/g d.w. [a]	%	ng/g d.w. [a]	%	ng/g d.w. [a]	%
nC14	Tetradecane	0.00 [b]	0.00 [b]	2.9	0.00 [b]	3.8	0.00 [b]
nC15	Pentadecane	3.8	0.00 [b]	5.0	0.00 [b]	5.5	0.00 [b]
nC16	Hexadecane	5.9	0.01	6.3	0.01	12.4	0.01
nC17	Heptadecane	29.5	0.03	39.5	0.03	43.8	0.03
nC18	Octadecane	19.6	0.02	44.5	0.04	15.9	0.01
nC19	Nonadecane	52.4	0.06	113.7	0.10	205.8	0.14
nC20	Eicosane	23.9	0.03	28.2	0.02	26.3	0.02
nC21	Heneicosane	548.5	0.59	662.2	0.53	573.2	0.40
nC22	Docosane	369.9	0.43	402.2	0.36	399.9	0.29
nC23	Tricosane	24,937.1	27.01	37,161.9	32.95	38,767.9	27.26
nC24	Tetracosane	8611.0	0.93	1166.3	1.03	1215.5	0.85
nC25	Pentacosane	29,576.5	32.07	45,302.3	40.17	4105.2	38.04
nC26	Hexacosane	780.4	0.85	885.0	0.78	1039.9	0.73
nC27	Heptacosane	14,193.2	15.39	12,732.3	11.29	23,306.5	16.39
nC28	Octacosane	489.0	0.53	371.3	0.34	618.4	0.43
nC29	Nonacosane	5277.4	10.62	7113.6	6.31	10,959.3	7.70
nC30	Triacontane	353.1	0.38	385.6	0.30	446.7	0.31
nC31	Hentriacontane	9729.5	10.55	5353.0	4.75	8977.0	6.30
nC32	Dotriacontane	175.6	0.19	124.2	0.11	153.2	0.11
nC33	Tritriacontane	256.1	0.28	916.5	0.81	1362.4	0.96
Total ng/g		92,209		112,777		142,234	
CPI_{25}		29.6		33.0		36.1	

[a] d.w. = dry weight; [b] < 0.005%.

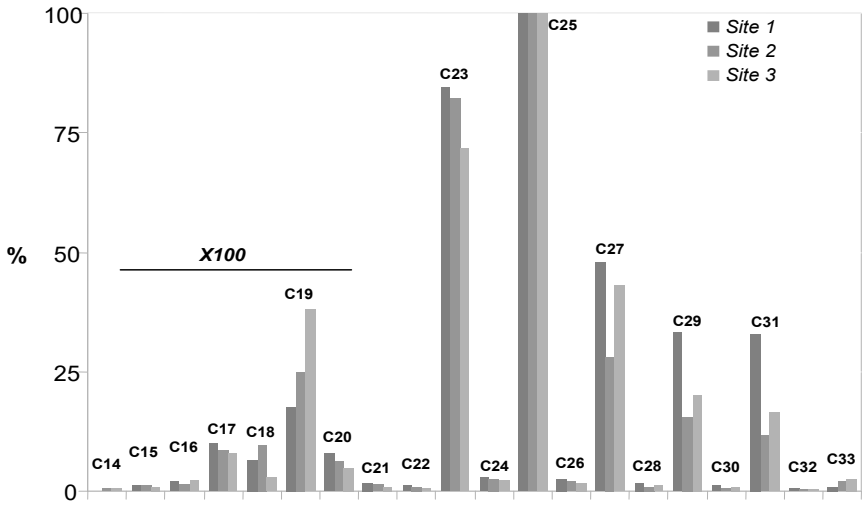

Figure 2. Comparative n-hydrocarbon distributions (percent contribution vs. carbon number) for individual homologs in the monitoring honeybee samples (data normalized to the nC25). ×100 = values multiplied by 100.

The components of this class were between 14 and 33 carbon atoms, with a predominance of long-chain congeners having odd carbon numbers. Neither in bee samples of the impacted villages nor of background remote areas were substantial qualitative differences observed. Aside from total concentrations showing mono-modal distribution with loadings ranging from 92.2 to 142.2 μg/g d.w., in all samples, the *n*C23 and 25 congeners were the most abundant with a contribution of ~30 and 40%, respectively, Table 1.

*n*C27, 29, and 31 were the other major congeners. In contrast, the sum of those with an even number of carbon atoms (*n*C14–32) contributed only 3.0% to the total concentration. The prevalent profile is consistent with typical biogenic sources, probably originating from the simultaneous contribution of primarily bee cuticular lipids and plant wax aerosol coming into contact with the insects during foraging flights. The estimated CPI_{25}, being equal to or exceeding 30, would confirm that. Strong biogenic emissions of higher land plants, in fact, are identified by values significantly higher than 5, concomitant with the predominance of the C27 and 29 homologs [37], while anthropogenic associated sources < 1 [36]. Considering that in all investigated samples paraffinic profile exhibited an apex on *n*C25, the contribution to biogenic input from bee cuticular lipids would appear prevalent. In addition, as reported in the available technical literature on chemical communication of social insects, the *n*-alkane homologs less than C23 are not observed [38–40]. Then, in our monitoring samples, paraffins between *n*C14 and 20 should probably be linked to the anthropic pollution without no relevant interferences from insect cuticular secretions, with an average concentration of 229.6 ± 89.7 ng/g honeybee d.w. In all the cases, comparisons of the anthropic contents of affected sites (1 and 2) to those of remote reference area (3), as well as for total n-alkane loadings, showed a consistent increase in their concentrations and not the opposite, as can be expected. This may be due to a major contribution in rural and remote aerosols of organic carbon from flora (>*n*C15), thus overlapping and altering the anthropogenic hydrocarbon profile in analyzed monitoring honeybees.

3.3. Polycyclic Aromatic Compound Fraction

The attribution of emission source in monitoring honeybees caught close to the two little villages and "uncontaminated" reference site, through the qualitative profile of polycyclic aromatic compounds (PACs), highlighted the primary presence of PAHs, then of their sulfur heterocycle analogs (PASHs), as well as alkylated and oxygenated derivatives. This last fraction was numerically fewer than related parents and not the aim of this dissertation. At locality 1, the foraging bees were particularly contaminated compared to those at the other two investigated sites (2 and 3). In this sample, 177 compounds were positively identified, as reported in detail in Table 2, a number not less than that found in PM10 characterization (200 PACs) [41].

For a more comprehensive understanding, the present study was not only addressed to quantify 16 priority EPA-PAHs, but also triphenylene, benzo(*ghi*), (*a*) and (*j*)fluoranthene, benzo(*e*)pyrene, indenofluoranthene, dibenzo(*a,c*)anthracene, benzo(*b*)crysene, coronene, and isomers (*a,l*), (*a,e*), (*a,i*) and (*a,h*) of dibenzopyrene. On the other hand, some of the latter have higher toxicological equivalence factors than benzo(*a*)pyrene [42]. Alkylated fraction of PAHs showed from mono- and poly-methylated naphthalenes to monobenzo(*a*)fluoranthene-perylenes. In the foraging honeybees of locality 2 and the reference area, the alkylated PAHs were less numerous and reduced to only phenanthrene-anthracene parents. Therefore, PASH fraction highlighted the presence of trimethyl benzothiophene, dibenzothiophene (DBT), nine dimethyl derivatives, two isomers of benzonaphthothiophene (1,2-*b*), (2,1-*b*) (BNT), two mono- and five di-methyl derivatives, one mono-methyl phenanthro(4,5-*bcd*)thiophene, and two of benzo[2,3]phenanthro(4,5-*bcd*)thiophene, as synoptically reported in Table 3.

In the foraging bee sample caught near locality 2 affected by emissions from both cement factories, only isomers of BNT and the di-methylated derivatives of DBT were present, whereas these last di-methylated isomers were the only sulfur heterocycle compounds identified in the reference not contaminated area (site 3). The parents of PAHs, essentially of the pyrolytic kind, contextually to their alkylated derivatives, result in being connotative of contribution to environmental pollution from petrogenic inputs and unburnt fossil fuels, too [43]. Although not a few km away from the emissive sources, on the Central Apennines, the monitoring bees of the reference area appear to be still polluted by the same pollutants found in contaminated sites, even if at lower amounts, as shown in Table 4.

Table 2. Identity list of PACs in honeybee sample from monitoring station 1 and related chromatographic retention times (Rt).

Peak nr	Compound	Rt	Peak nr	Compound	Rt
1	Naphthalene	5.080	76	x,y-di-methyl Phen/Anthr	15.386
2	2-methyl-Naphthalene	6.316	77–78	x,y-di-methyl Dibenzotiophene	15.539–15.554
3	1-methyl-Naphthalene	6.518	79	x,y-di-methyl Phen/Anthr	15.658
4	1,1′-Biphenyl	7.318	80–81	x,y-di-methyl Dibenzotiophene	15.673–15.690
5	Biphenyl-3-ol	7.535	82–83	x,y-di-methyl Phen/Anthr	15.758–15.842
6–9	x,y di-methyl-Naphthalene	7.651–8.064	84	tetra-methyl-s-Indacene-1,7-dione, tetra-hydro-	15.975
10	Acenaphthene	8.212	85	x,y-di-methyl Phen/Anthr	16.009
11	2-acetossi-2-metossi-Biphenyl	8.462	86	Biphenyl-2-ol-5, 1,1-dimethyletil-	16.093
12	Acenaphthylene	8.829	87–89	x,y-dimethyl Phen/Anthr	16.126–16.310
13	x,y,z tri-methyl Naphthalene	8.829	90	Fluoranthene	16.545
14	x,y,z tri-methyl Naphthalene	8.927	91	x,y-di-methyl 9,10-Anthracenedione	16.562
15	x,y,z tri-methyl Benzo(*b*) thiophene	8.993	92	2-Phenylmethyl Naphthalene	16.819
16	Dibenzofuran	9.061	93–95	x,y,z-tri-methyl Phen/Anthr	16.869–17.022
17–21	x,y,z tri-methyl Naphthalene	9.061–9.542	96	x,y-di-methyl Phenanthrene, diidro-	17.022
22	x-y di-methyl Biphenyl	9.774	97	x,y,z-tri-methyl Phen/Anthr	17.105
23	Fluorene	9.873	98	x,y-di-methyl 9,10-Anthracenedione	17.139
25	x methyl Biphenyl	10.041	99	x,y,z-tri-methyl Phen/Anthr	17.190
26	x,y di-methyl diphenyl Methane	10.124	100	x,y,z-tri-methyl Phen/Anthr	17.256
27	x,y,z,t tetra-methyl Naphthalene	10.190	101	Pyrene	17.305
28	x,y di-methyl Biphenyl	10.307	102–106	x,y,z-tri-methyl Phen/Anthr	17.322–17.657
29	Benzophenone	10.374	107–110	x,y,z,t-tetra-methyl Phen/Anthr	17.740–18.090
30–31	x,y di-ethyl Biphenyl,	10.524–10.557	111	Methylethyl Benzo(*a*)acridine	18.225
32	x,y,z,t tetra-methyl Naphthalene	10.640	112–113	x-methyl Fluoranthene/Pyrene	18.192–18.275
33	diisopropyl Naphthalene	10.756	114	3-methyl Phenanthro(4,5-*bcd*) tiophene	18.357
34	Nitrophenol, ditert-buthyl-	10.822	115–116	x-methyl Fluoranthene/Pyrene	18.492–18.610
35	Biphenyl, di-ethyl-	10.873	117	Triphenyl methane	18.696
36	Nitrophenol, diter-buthyl-	10.956	118	3-methyl Phenantro(4,5-*bcd*) tiophene	18.749
37–38	Biphenyl, di-ethyl-	11.005–11.256	119–131	x-methyl Fluoranthene/Pyrene	18.850–20.861
39–41	diisopropyl Naphthalene	11.407–11.541	132	Benzo(*a*)nafto(2,1-*d*)tiophene	20.911
42	Ethane-1,1-bis(p-ethylphenyl)	11.608	133	x,y-dimethyl Fluoranthene/Pyrene	20.945
43	2-vinyl-2,3-di-hydro Nahthofurane	11.675	134	Benzo(*ghi*)fluoranthene	20.978
44	Benzene-1,1-methylene bis(4-methyl)	11.692	135	Benzo(*a*)naphtho(1,2-*d*)tiophene	21.213
45	1,3 Pentadiene, 1,1-di-phenyl	11.726	136	Benzo(*a*)anthracene	21.831
46	1,2-dimethyl Naphtho(2,1-*b*) furane	11.825	137	Crisene+Trifenilene	21.984

Table 2. *Cont.*

Peak nr	Compound	Rt	Peak nr	Compound	Rt
47	Biphenyl, di-ethyl-	11.859	138	6-methyl Benzo(*b*)nafto(2,3-*d*) tiophene	22.198
48	x,y,z,t-tetramethyl Biphenyl	11.875	139	8-methyl Benzo(*b*)nafto(2,3-*d*)tiophene	22.533
49	Benzaldeide-3,5-di-tert-buthyl -4-hydrossi	11.926	140–141	1-H-Indene-2,3-diidro-trimethylphenil	22.600–22.746
50	4-methyl Acridone	11.976	142–147	x-methyl Benzo(*a*)anthr/Crisene	23.297–23.863
51	Anthracene, tetrahydro-9-propyl	11.976	148–152	x,y-di-methyl Benzo(*b*)naphtho (2,3-*d*)tiophene	23.647–24.346
52	10-methyl Acridone	12.075	153–155	Benzo(*b,j,k*)fluoranthene	25.740–26.012
53	Biphenyl, di-ethyl-	12.125	156	Benzo(*a*)fluoranthene	26.162
54	Dibenzotiophene	12.175	157	Benzo(*e*)pyrene	26.162
55	x,y,z-trimethyl-3-phenyl di-hydro indene	12.209	158	Benzo(*a*)pyrene	26.803
56	Phenanthrene	12.557	159	Perilene	27.063
57	Diphenoxyethane	12.641	160	5,8-di-methyl Benzo(*c*)phenanthrene	27.342
58	Anthracene	12.723	161	methyl Benzo[2,3]phenanthro (4,5-*bcd*)tiophene	27.392
59	Fluorenone	12.756	162	3-methyl Benzo(*j*)aceantrilene	27.880
60	1-Indene-2,3-di-hydro-1,1,3-trimethyl-3-phenyl	12.906	163–166	x-methyl Perilene/Benzo [1,2-*b*:4,3-*b*] ditiophene-1-Phenyl	27.998–28.387
61	9,10-Anthracenedione	13.635	167	Indenofluoranthene	29.763
62	x-methyl Phen/Anthr	13.788	168	Indeno(1,2,3-*cd*)pyrene	31.314
63	9,10-Anthracenedione, 2-ethyl	13.804	169–170	Benzo(*ac*+*ah*)anthracene	31.506
64	9-Amminofluorenone	13.957	171	Benzo(*b*)crisene	31.906
66	2-methyl Phenanthrene	14.207	173	Dibenzo(*a,l*)pyrene	34.497
67	1-methyl Phenanthrene	14.360	174	Dibenzo(*a,e*)pyrene	35.353
68	x-methyl Phen/Anthr	14.462	175	Coronene	39.390
69	Dimethyl carbazole	14.513	176	Dibenzo(*a,h*)pyrene	39.670
70	9-methyl Anthracene	14.548	177	Dibenzo(*a,i*)pyrene	43.040
71–75	x,y-dimethyl dibenzotiophene	14.785–15.335			

x,y,z,t refer to the indeterminate position of methyl substitution; Phen = phenanthrene; Anthr = anthracene.

Table 3. Concentrations of PASHs parent and alkylated (expressed as ng/g honeybee d.w).

		Monitoring Site		
		1	2	3
	Isomer nr	ng/g d.w. [a]		
trimethyl Benzothiophene	1	0.05	<0.03	<0.03
Dibenzothiophene	1	4.36	1.15	1.55
dimethyl Dibenzothiphene	9	8.24	6.01	<0.03
Benzonaphtho(2,1-*d*)thiophene	1	3.55	<0.04	<0.04
Benzonaphtho(1,2-*d*)thiophene	1	0.73	<0.04	<0.04
methyl Benzo(*b*)naphthothiophene	2	8.98	<0.05	<0.05
dimethyl Benzo(*b*)naphthothiophene	5	5.15	<0.05	<0.05
methyl Phenanthrothiophene	1	2.46	<0.05	<0.05
methyl Benzo(*b*)phenanthrothiophene	2	2.11	<0.05	<0.05
Total PAHs ng/g d.w. [a]		35.63	7.16	1.55
ng/g w.w. [b]		13.50	2.72	0.41

[a] d.w. = dry weight; [b] w.w. = wet weight.

Table 4. PAH concentrations (expressed as ng/g honeybee d.w.) of three monitoring bee samples.

	Monitoring Site		
	1	**2**	**3**
Compound	**ng/g d.w. [a]**		
Naphthalene *	33.63	19.67	31.97
Acenaphthylene *	0.81	0.74	0.98
Acenaphthene *	1.69	2.41	2.26
Fluorene *	40.89	107.35	92.88
Phenanthrene *	29.84	25.16	21.49
Anthracene *	10.31	2.87	3.10
Fluoranthene *	7.03	9.58	9.10
Pyrene *	16.65	7.75	6.13
Benzo(*ghi*)fluoranthene	0.61	<0.04	0.70
Benzo(*a*)anthracene *	NQ	NQ	NQ
Chrisene+Triphenylene *	24.92	11.62	9.15
Benzo(*b*+*k*+*j*)fluoranthene *	9.36	<0.05	<0.05
Benzo(*a*) fluoranthene	1.25	<0.08	<0.08
Benzo(*e*)pyrene	17.79	<0.08	<0.08
Benzo(*a*)pyrene *	24.91	<0.08	<0.08
Indenofluoranthene	4.40	<0.08	<0.08
Indeno(123-*cd*)pyrene *	26.12	<0.10	<0.10
Dibenzo(*ac*+*ah*)anthracene *	6.68	<0.10	<0.10
Benzo(*b*)chrysene	19.93	<0.10	<0.10
Benzo(*ghi*)perylene *	28.51	<0.10	<0.10
Dibenzo(*al*+*ae*+*ai*+*ah*)pyrene	8.79	<0.40	<0.40
Coronene	27.67	<0.10	<0.10
Total PAHs ng/g d.w. [a]	341.77	187.14	177.67
ng/g w.w. [b]	129.95	71.15	67.50

[a] d.w. = dry weight; [b] w.w. = wet weight; NQ = not quantified; * = US-EPA priority.

This suggests that a pristine area, assumed as unpolluted, can still be affected by chemicals that can cover long distances from the emission sources through the atmosphere by cold trapping or long-range transport phenomena before being deposited [44,45]. In this respect, the regional orography contributes to the diffusion of pollutants in a given territory too. Analogous considerations are valid concerning the fraction of sulfur heterocyclic found in all samples examined. Additionally, this class of compounds, likewise for PAHs, may have two origins: petrogenic and pyrolytic [46]. Sulfur lighter derivatives (one or two rings) are equally generic markers of petrogenic and pyrolytic sources, whereas higher derivatives (three to five rings) come from coal and diesel combustion [47] and, in some instances, as specific products of tires combustion [48]. However, homologs at higher molecular weight, such as phenanthro(4,5-*bcd*)thiophene and benzo(2,3)phenanthro(4,5-*bcd*)thiophene and its alkylated derivatives, are reported as typical coal combustion markers [49,50], with probable carcinogenic and mutagenic higher activity exceeding that of benzo(*a*)pyrene [51,52], as well as for methylated isomers of BNT [53]. This evidence would confirm the presence of these molecules, referable not only to the burning of coal and heavy oil, but also to the incineration of tire carcasses by cement plants.

From a quantitative point of view, concentrations of PAHs ranged from 177.67 to 341.77 ng/g d.w. (Table 4) and, as noted earlier, with maximum contamination in the sample of locality 1. In this sampling site, comparable intake was observed for PAHs present in suspended aerosol as gas phase (low molecular weight) and as adsorbed on particulate matter (high molecular weight). As it is common knowledge, partitioning and residence time in the atmosphere of compounds such as PAHs are mainly a function of weather conditions (i.e., temperature, windiness, rainfall) and of intrinsic physical-chemical properties of single analytes (i.e., vapor pressure, melting and boiling point, density) as well as from molecular weight. The other monitoring site and that of the pristine reference area presented loadings of the same order of magnitude, with almost exclusive contribution

from aromatic hydrocarbons at the lower molecular weight. With respect to the work by Perugini and co-workers [25], where PAHs never exceded the 10 ng/g w.w. without a substantial difference between urban areas and wildlife reserves, our analytical data were higher. In addition, heavier homologs had never been detected by this author. On the opposite hand, in a not formally published paper, ref. [54] reported concentrations close to 2 ng/g d.w. just for benzo(*a*)pyrene, emphasizing a positive correlation between insects and sites at different pollution levels, while another work by the same author [33] reported benzo(a)pyrene concentration values higher than 5 ng/g d.w. in two monitoring stations near the two emission sources also studied by us. A later study case by Kargar and co-workers [28] has shown total PAH values comparable to ours, 261.18–553.33 ng/g d.w., but with a lower benzo(*a*)pyrene content, 0.03–1.32 ng/g d.w.

Neither for PASHs nor for their alkylated homologs could a qualitative and quantitative comparison with the literature be made, as no such data exist at present. Amounts of these pollutants, in all examined matrices, ranged from 1.55 to 35.63 ng/g d.w., matching the positive trend of contamination degree (Table 3) and magnitude versus PAH concentrations as usually found in atmospheric particulate matter. The relevant contribution of alkylated derivatives to the total loading (≥30%) reflects even more petrogenic input to environmental pollution.

Contextually, for a more correct attribution of polluting sources, semi-quantitative forensic techniques (diagnostic ratios) are tentatively used. Analysis of PAH diagnostic ratios would seem to confirm qualitative data to identify regional point sources from the industrial district, Table 5.

Table 5. –PAH diagnostic ratio of the three monitoring bee samples.

	Monitoring Site			Literature	
Diagnostic Ratio	**1**	**2**	**3**	**Pyrolitic**	**Petrogenic**
Low/High	0.5	3.9	4.4	<1	>1
Ph/An	2.9	8.8	6.9	<10	>15
Fl/Pyr	0.4	1.2	1.5	>1	<1
Fl/Fl+Pyr	0.3	0.6	0.6	>0.5	<0.5
An/Ph+An	0.26	0.10	0.13		
IP/IP+BghiP	0.48	-	-		
				fresh	**aged**
BeP/BaP	0.71	-	-	<1	>1

Low = phenanthrene + anthracene + fluoranthene + pyrene; High = chrysene + triphenylene + benzo(b+j+k)fluoranthene + benzo(e) pyrene + benzo(a)pyrene + indeno (123-cd)pyrene + benzo(ghi)perylene; P = phenanthrene; A = anthracene; Fl = fluoranthene; P = pyrene; IP = Indeno(123-cd)pyrene; BghiP = Benzo(ghi)perylene; BeP = Benzo(e)pyrene; BaP = Benzo(a)pyrene.

In fact, some index values between congeners with the same molecular weight and different thermodynamic stabilities can determine whether the pollution was generated from petrogenic and pyrolytic sources or from a variable combination of both. As widely documented, some isomers are steadier than others, and their predominance reveals a pyrolytic kind, while those less stable are petrogenic [55]. In all the cases, the concentration ratio of phenanthrene vs. anthracene (Ph/An < 10) provided a realistic indication of combustion origin, as well as for that one of Low vs. High in honeybees of locality 1 and fluoranthene vs. pyrene (Fl/Pyr) and fluoranthene vs. sum of fluoranthene and pyrene (Fl/Fl+Pyr) in locality 2 and in reference area [56,57]. To this input would seem added that petrogenic one, as it is evident from data shown in Table 5, Low/High > 1 for locality 2 and reference area and Fl/Pyr < 1 and Fl/Fl+Pyr < 0.5 for locality 1. Considering that pollutant profiles are a snapshot of the species' lifetime and of their chemical alteration in the atmosphere as a result of dynamics of short-term fate, weathering, ultraviolet photoreactions, and gas-phase/particle partitioning, the calculated indices would seem ambiguous and disagreeing among beehives impacted from similar emissive sources. In this context, then, this evidence can be seen as realistically related to 'ageing' of pollution

too. Honeybees living in the surroundings of locality 1 would seem impacted by 'fresh' pollution before bee sampling, as also supported by the ratio between the two isomers of benzopyrene, (e) and (a), <1 [58] and phenanthrene and anthracene (2.9), much lower compared to other two monitoring hives (8.8 and 6.9). Conversely, in these last monitoring stations was found 'aged' contamination with evident degradation of photochemical less stable isomers, anthracene, and pyrene and permanence in the atmosphere of more volatile aromatic hydrocarbons preferentially partitioned to the gas phase. In addition, the ratio of Fl/Fl+Pyr close to 0.6 in these two samples confirmed the contribution to emissions of gasoline combustion [59].

For further proof of source apportionment, the cross plot of diagnostic ratios between An/An+Ph and Fl/Fl+Pyr confirmed contextually mixed petroleum combustion/pyrolytic and petrogenic inputs, in locality 2 (0.10 vs. 0.55) and reference area (0.13 vs. 0.60) samples, respectively. Instead, values of Fl/Fl+Pyr vs. IP/IP+B*ghi*P (0.3 vs. 0.48) positioned the pollution profile of locality 1 biomonitors to the border line from petroleum and coal combustion [60].

4. Conclusions

Comprehensive analysis of organic extractable compounds in foraging honeybees, on the return to their hives, underline and confirm the ability of these insects to be environmental sentinels of a given geographic area, providing useful information on pollution origin and contamination levels.

However, the small number of bee pools, for ethical reasons, does not invalidate the findings made in this respect, as they were obtained from a statistically significant sample in terms of the numbers of insects and the representativeness of hive locations.

Unlike what is usual in environmental investigations, the study of the composition of aliphatic hydrocarbon mixtures on honeybee bodies does not allow us to identify unambiguously the apportionment of different inputs, and to ascribe quantitatively to the respective sources. This is particularly true when a preponderant contributor is present, such as insect epicuticular wax.

On the contrary, the distribution and concentration of PAHs, PASHs, and their alkylated derivatives in samples from different monitoring stations allowed us to trace and identify the nature and sources of contaminants from overall profiles. These findings were also achieved, through the analysis of diagnostic molecular ratios contextually combined with qualitative criteria, confirming the honeybee's ability to reflect PACs pollution despite the distance from potential point sources.

However, it should be noted that only a combined approach ensures an unambiguous assignment of realistic pollution sources in giving monitoring area. In this specific research field, the literature report studies only a single class of PACs, always the PAHs, and a restricted number of its components, usually referred to as those provided for by the health reference regulations of different countries. These few compounds would not always allow discrimination of the pollution sources correctly, as reported in this paper.

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