Heavy metal bioaccumulation in honey bee matrix, an indicator to assess the contamination level in terrestrial environments*

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A B S T R A C T

The most significant risk factor for organisms living in an environment contaminated by heavy metals is the metal bioavailability. Therefore, an efficient ecotoxicological approach to metal contamination is the measure of bioaccumulation level in target organisms. In this work, we characterized the heavy metal bioaccumulation in honey bees, *Apis mellifera ligustica*, collected at 35 sites from Umbria (Central Italy). The comparison of our data with selected Italian investigations revealed metal bioaccumulation in honey bee matrix of the same order of magnitude, with Cd showing a higher variability. To generalize the results, we developed a Honeybee Contamination Index (HCI) based on metal bioaccumulation in honey bees. An application of the HCI to the present dataset revealed cases of low (sixteen sites), intermediate (eighteen sites), and high (one site) metal contaminations. The comparison of HCI values from the Umbrian dataset with values calculated for other Italian and European metadata showed that most of the Umbrian sites fell in the portion of low and intermediate contamination conditions. HCI represented a reliable tool that provided a piece of concise information on metal contamination in terrestrial environments. Parallel to this effort, we have determined, the metal concentrations in the airborne particulate matter (PM_{10}) at three regional background-monitoring stations in Umbria. These stations are representative of the average air quality of the areas of the investigated apiaries. A comparative analysis of metal enrichment factors in PM_{10}, and honey bees suggested that the contamination in the bees was related to the PM_{10} values only to a minor extent. On the other side, a clear enrichment of metals such as Cd, Mn, Zn, and Cu in the honey bees appeared to depend on very local conditions and was probably related to the use of pesticides and fertilizers, and the resuspension of the locally contaminated soils and agriculture residues.

1. Introduction

The emission from a variety of anthropogenic sources of heavy metals in the environment can directly contaminate living organisms (Alcorlo et al., 2006; Di Veroli et al., 2014; Goretti et al., 2018) or affect trophic networks in an indirect way (Filgueiras et al., 2002; Davutluoglu et al., 2011; Pallottini et al., 2015) up to cause the onset of pathologies also for humans (Hutton, 1987; Huff et al., 2007; Elia et al., 2018).

In recent decades, environmental contamination by heavy metals has become a topic of primary importance for ecosystems and human health (Wuana and Okieimen, 2011; Gall et al., 2015). Some heavy metals are essential for organisms, such as nickel, copper, zinc and chromium (a lack or an excess of them can cause the onset of diseases), while others are toxic even in traces, such as cadmium and lead (Keil et al., 2011; ATSDR, 2017).

However, it is difficult to directly correlate heavy metal levels in the environment with their impact on biota, due to the intrinsic complexity of toxic effects and due to ecosystem variability that conditions metal dispersion and entry into the food chain (Devi et al., 1996; Millàn et al., 2008).

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Indeed, the risk factor is due to the bioavailability of the metal itself and only indirectly to its absolute amounts in the environmental compartment. In this regard, the use of biological systems for environmental monitoring has the advantage of assessing the actual toxicity of contaminants on living organisms (Di Veroli et al., 2012).

An important role in bioindication of terrestrial environments is played by *Apis mellifera* Linnaeus, 1758 (Western honey bee) used in apiculture, chiefly *Apis mellifera ligustica* (Spinola, 1806), which reveals environmental degradation through various types of indicators such as the high mortality and the ability to accumulate contaminants in their tissues or in bee products (Crane, 1984; Devillers and Pham-Delègue, 2002; Bogdanov, 2006; Satta et al., 2012; Zhelyazkova, 2012; Badiou-Bénéteau et al., 2013; Krakowska et al., 2015; Van der Steen et al., 2016; Herrero-Latorre et al., 2017; Zhou et al., 2018).

Honey bees are social insects living in vast colonies of about 40,000 individuals, typically including a single queen, few males (less than 0.1% of the colony) and thus mostly females (workers), which are the smallest specimens of the colony. The workers are sterile and usually have a lifespan of about six weeks during the summer with different duties depending on their age. The older workers become forager bees and are designated to find pollen, nectar, propolis, honeydew and water.

The foragers can visit an average of 1,000 flowers in one day. From this, the 10,000 foragers present during the spring-summer period in a beehive, come to visit up to 10 million flowers daily (Leita et al., 1996) and require 3e4 million of flowers visited to produce half a kilogram of honey (Fakhimzadeh and Lodenius, 2000). Each forager makes 10e15 trips a day, it can fly at a speed of about 25 km h^{-1} (Dornhaus et al., 2006) and it has a flight radius, commonly within 1.5 km from the hive.

The search for food is operated with the help of highly developed sensory organs, as the compound eyes sensitive to the ultraviolet radiations reflected by the different flower colorings, and as the antennae equipped of different receptors for detecting the odors of flowers as well as the pheromones produced by the queen and by the workers that gives a specific smell to the colony. Honey bees, once identified a nectar source, communicate with each other, inside the hive, employing dances indicating the distance and direction of the food source to the other worker bees of the colony (Devillers, 2002). Therefore, each flowering present in the action range of the colony is assiduously followed by the foraging bees and if a set of colonies (apiary) is present in the same action radius, the plant species visited are the same for all the foragers of the apiary; this is why beekeepers are able also to produce monofloral kinds of honey (Gardi et al., 2015).

These ethological aspects allow the bees to come in contact with a large number of pollutants in all the environmental compartments (air, soil, vegetation and water) of an area, commonly around 7 km^2 surrounding their apiary (Devillers, 2002). Consequently, the bioaccumulation level of heavy metals in the tissues of forager bees is an efficient detector of environmental contamination by metals (Porrini et al., 2002; Van der Steen et al., 2012, 2015; Ruschioni et al., 2013; Gutiérrez et al., 2015; Zarić et al., 2017), transferred as particulate matter via the atmosphere (Ferrero et al., 2012; Negri et al., 2015; Federici et al., 2018) or as inorganic/organic fertilizers and pesticides that contain heavy metal contaminants (Mortvedt, 1995; Gupta et al., 2014; Roberts, 2014; Zarić et al., 2016).

The main objectives of the present work are: *(i)* the analysis of the bioaccumulation levels of heavy metals in forager honey bees, in different sites of Umbria Region (Central Italy) with a variable degree of anthropogenic impact; *(ii)* the definition of an index, Honeybee Contamination Index (HCI), which can express in a concise and straightforward manner the contamination level of a single or a set of heavy metals; *(iii)* the comparison of HCI values calculated for the Umbrian dataset with metadata found in literature (Italy and Europe);*(iv)* the identification of the sources and mechanisms of metal contamination in honey bees by comparison of enrichment factors in airborne particulate matter (PM_{10}) and honey bees.

2. Materials and methods

2.1. Honey bee sampling campaign

The survey was carried out during the spring-summer period (2014e2015) in various parts of the Umbria Region and considered 35 apiaries (Fig. 1, Table S1 in Supplementary Material). The sampling sites were selected by the different degree of environmental pollution, in particular, due to agricultural and industrial activities.

All the honey bee samples were collected after having acquired the beekeepers' consent to participate anonymously to the present study. Honey bee sampling was carried out according to the safety rules and in respect of the colonies, avoiding to open the hives and trying not to disturb the insect activity. The collection concerned forager bees, i.e., those bees collecting nectar and pollen, which are more easily exposed to environmental contamination.

For each apiary, about 100 forager bees were taken from the central hives (3/4 hives) as a representative sample of the sampling site. Honey bee samples were pre-refrigerated on site. In the laboratory the samples were initially stored in a freezer at −20 °C; once death was determined they were divided into sub-samples, transferred to Petri dishes and stored at −80 °C in order to guarantee the correct preservation of samples. Before being submitted to the analytical procedures, each honey bee specimen was cleaned from the pollen that could be present on the pollen baskets of the hind legs and eventual parasites, i.e., *Varroa destructor* (Anderson & Trueman, 2000), were removed.

2.2. Particulate matter sampling campaign

The particulate matter (PM_{10}) sampling campaign was conducted at two urban background stations located in the main Umbrian cities Perugia (PGC) and Terni (TRC) and at the regional background station of Martano Mountain (MM) (Fig. 1). Perugia is the capital town of Umbria and the air quality is mainly influenced by vehicular traffic. On the other side, the primary sources of particulate matter in the Terni basin are the industrial emissions, in particular, stainless steel production. This source impacts the air quality in all the Terni basin area (Moroni et al., 2012; Ferrero et al., 2014). The MM station is the regional background station and is particularly suited to study long-range transport of air masses (Moroni et al., 2015; Petroselli et al., 2018, 2019). The station is affiliated to the EMEP (https://www.emep.int), and WMO SDS-WAS (https://sds-was.aemet.es) monitoring networks. In the present work, we consider yearlong campaigns conducted in 2013e2014 in PGC (24 samples) in the 2013e2016 at MM (48 samples) and in the 2015e2016 in TRC (73 samples). The samplings were conducted at the three stations with high volume PM_{10} impactors (TISH, TE6001) operated at a flux of 1140 L min^{-1} on quartz fiber filters (Whatman QMA 20.3*25.4 cm) for 24 h sampling. The samplings were distributed equally during the seasons trying to select different weekdays and including weekends.

2.3. Heavy metals analysis

For each honey bee sample, the heavy metal determination was carried out in two analytical tests on wet weight of about 1.0 g (13

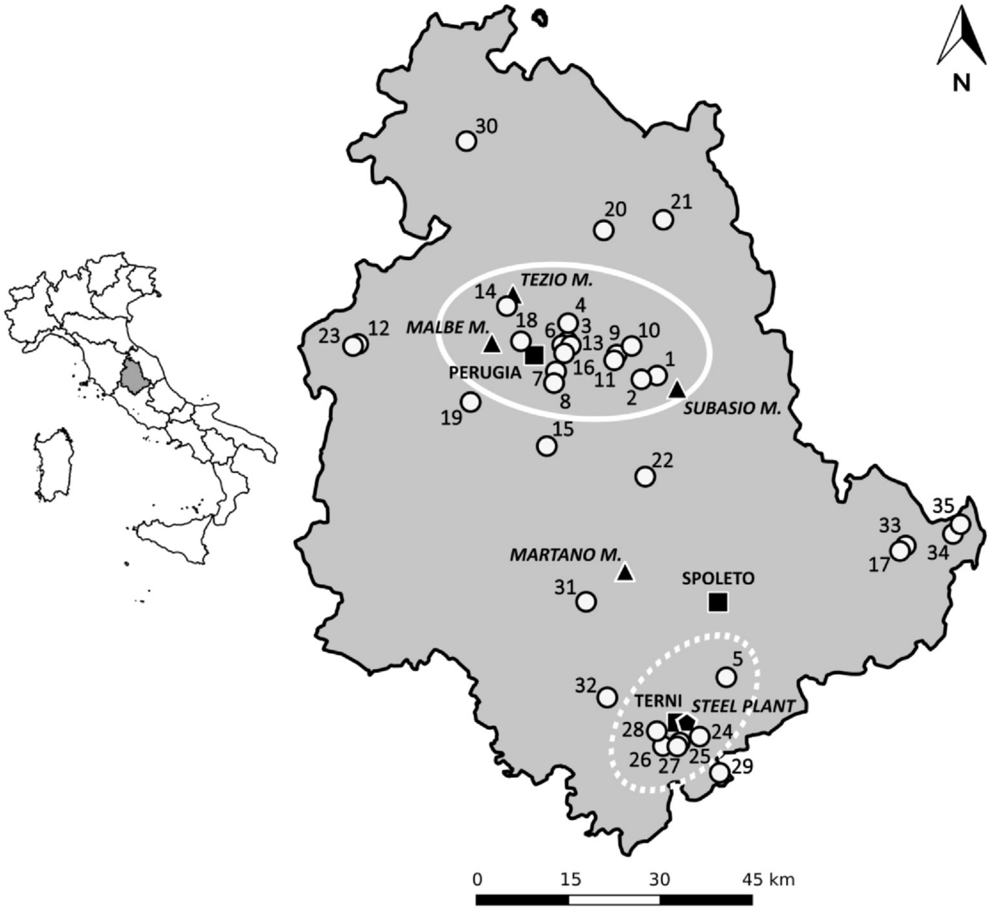

Fig. 1. Location of the thirty-five sampling sites (apiaries) - Umbria Region, Italy. Solid line indicate Perugino area, surrounding Perugia city; dashed line indicate Ternano area, surrounding Terni city. Squares, triangles and pentagons indicate cities, mountains and steel plant, respectively.

specimens in mean) corresponding to a dry weight of about 0.3 g. Honey bee samples were dried in the oven for 16 h at 105 °C. The acid digestion was carried out adding to the samples 8 ml of ultrapure nitric acid (65% v/v) and 2 ml of ultrapure hydrogen peroxide (30% v/v). The mixture was warmed up to 170 °C in a microwave digestion system for 30 min (MARS 5 Microwave Digestion System, CEM Corporation). After the digestion, the mixture was cooled, filtered (Whatman Grade No. 42, particle retention 2.5 mm) and ultrapure water was added to reach the volume of 25 ml.

Concentrations of heavy metals (Cd, Pb, Cr, Ni, Zn, Cu, Mn, and Fe) in the samples were determined by inductively coupled plasma optical emission spectrometry (ICP-OES Ultima 2, HORIBA Scientific) equipped with an ultrasonic nebulizer (CETAC Technologies, U-5000AT). The following wavelength lines (nm) were used: Cd, 214.438; Pb, 220.353; Cr, 267.716; Ni, 216.556; Zn, 213.856; Cu, 327.396; Mn, 257.61; Fe, 259.94. Commercially produced (ICP multi-element standard solution IV CertiPUR®, VWR Merck Chemicals, and Reagents) standard solutions (1000 mg l^{-1}) in nitric acid were used to prepare appropriate elemental calibration standards. Detection limits of the method (in mg kg^{-1}) were: Cd, 0.027; Pb, 0.131; Cr, 0.070; Ni, 0.082; Zn, 0.070; Cu, 0.080; Mn, 0.021; Fe, 0.026. The accuracy of the analytical method was controlled by BCR-185R bovine liver reference materials (the trace metals recovery were in the range of 80%e120%).

In the case of PM_{10} samples, a portion of the filter was digested in a microwave oven (START E Milestone, Italy) in Teflon vessels with 8 ml of ultrapure nitric acid (65% v/v) and 2 ml ultrapure hydrogen peroxide (30% v/v) (UNI EN 14902:2005 standard). The digested solution was diluted to 50 ml with ultrapure water and stored at 4 °C in PTFE bottles.

Elemental analysis of Cd, Pb, Cr, Ni, Zn, Cu, Mn, and Fe was performed using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, 7500 C Agilent, USA). Filter blanks were prepared and analyzed together with the samples, following the same procedures, and the amounts obtained were routinely subtracted from those of the samples. Detection limits of the method (in ng m^{-3}) were 0.012 for Cd and Pb; 0.060 for Cr, Cu, and Mn; 0.09 for Ni, Zn, and Fe. The quality and accuracy of quantitative analysis were routinely checked analyzing the NIST SRM 1648 standard for air particulate. The trace metals recovery was in the range of 80%e 110%.

2.4. Honeybee Contamination Index

The Honeybee Contamination Index (HCI) has been developed to assess heavy metal contamination in terrestrial environments through the use of *Apis mellifera ligustica* as bioindicator. HCI is the result of the combination of HCI_1 and HCI_2 that allows to define the metal contamination level in honey bees: *i)* H, a high contamination level when HCI_1 is positive; *ii)* L, a low contamination level when HCI_2 is negative; *iii)* I, an intermediate contamination level when HCI_1 is negative and HCI_2 is positive.

HCI_1 and HCI_2 are calculated using the following equation:

$$HCI_i = \log(C_{bees} / C_{bees\_i})$$

where: *i)* (C_{bees}/C_{bees_i}) is the ratio between the heavy metal concentration in honey bees (C_{bees}) and the relative threshold (C_{bees_i}) differentiated for high (C_{bees1}) and low (C_{bees2}) reference thresholds of contamination, respectively; *ii)* the threshold values for each heavy metal in honey bees were defined and reported by DiSTAL – UniBo (2010) and Gutiérrez et al. (2015), by setting the values of Cd, Pb, Cr, and Ni with 0.1, 0.7, 0.12, and 0.3 mg kg^{-1} for C_{bee1} and with 0.05, 0.3, 0.04, and 0.1 mg kg^{-1} for C_{bee2}, respectively. These threshold values are expressed in wet matter and converted to dry matter by considering a weight loss of 68% due to the drying process (Van der Steen et al., 2012). The same threshold values were used in Spain from Gutiérrez et al. (2015) and very similar levels in Netherland from Van der Steen et al. (2016).

In *Apis mellifera*, the uptake of non-essential metals (as Cd and Pb), for which no metabolisms are involved, is prone to rise reflecting the increase in environmental concentrations of these heavy metals, regardless of a particular threshold level (Di et al., 2016). However, a recent study on honey bees showed that also essential microelements (as Ni and Cr) for which regulatory mechanisms exist in order to prevent tissue metal load, may be accumulated at higher environmental levels (Giglio et al., 2017).

This index was calculated for each metal independently (HCI) and as the mean value of the individual HCI (HCI_{mean}) relative to the set of metals considered. This HCI_{mean}, expressed as a dimensionless value in a logarithmic scale, characterized on the whole the honey bee contamination level of each apiary for the considered set of metals.

HCI was calculated for honey bee dataset in Umbria (Central Italy) and applied to metadata found in the literature (from Italy and Europe).

3. Results

3.1. Heavy metal bioaccumulation and Honeybee Contamination Index (HCI)

The heavy metal investigation involved 35 honey bee colonies bred in hives distributed throughout the Umbria Region territory. The heavy metal concentrations in honey bees are reported in Table 1. Cd was the metal with the highest variability while Cr, Zn, Cu and Fe showed the lowest variability. Overall concentrations tended to increase in the Southern-Eastern part of the region (see Supplementary Material, Figs. S1a–h shows the relative maps of metals in the 35 sampling sites).

The definition of high and low reference thresholds of the bioaccumulation level in honey bee matrix for Cd, Pb, Cr, and Ni (DiSTAL – UniBo, 2010; Gutiérrez et al., 2015) allowed applying the Honeybee Contamination Index (HCI) that expressed an indication on metal contamination of terrestrial environments.

Cadmium HCI showed a level of low contamination at twenty-two sites, intermediate contamination at three sites (9, 19 and 25) and high contamination at the remaining ten sites (in particular at site 5) (Fig. 2a). Lead HCI showed a level of low contamination at thirty-two sites and intermediate contamination at the remaining three sites (17, 21 and 8) (Fig. 2b). Chromium HCI showed a level of low contamination only at one site (33), intermediate contamination at twenty-seven sites and high contamination at the remaining seven sites (in particular at site 28, 25, and 34) (Fig. 2c). Nickel HCI showed a level of low contamination at fifteen sites (central part of the Region), intermediate contamination at four sites (27, 25, 7 and 30) and high contamination at the remaining sixteen sites (in particular at sites 5 and 19) (Fig. 2d). In the Supplementary Material, Figs. S2a–d shows the HCI maps for Cd, Pb, Cr and Ni in the 35 sampling sites.

Finally, HCI_{mean} application, as the mean value of the four heavy metal considered, allowed to express a synthetic judgment (Fig. 3a). A low contamination condition was detected at sixteen sites (in particular at site 13), an intermediate contamination condition at eighteen sites and a high contamination condition only at one site (site 5). Interestingly sites of low and intermediate contamination conditions were often geographically very close to each other and distributed in a somewhat irregular pattern in both the Perugino and Ternano areas (Fig. 3b).

Table 1
Honey bee heavy metal concentrations (Cd, Pb, Cr, Ni, Zn, Cu, Mn, and Fe) in the thirty-five sampling sites (dry weight, mg kg^{-1}): mean, minimum (min), maximum (max), standard deviation (s.d.), first quartile, third quartile, median values and limit of detection (LOD).

Sites	Cd	Pb	Zn	Cr	Ni	Cu	Mn	Fe
1	0.15	0.32	115.92	0.19	0.08	14.78	53.11	62.81
2	0.36	0.30	171.34	0.22	0.08	16.62	75.57	80.67
3	0.14	0.21	87.91	0.17	0.08	11.62	55.27	75.72
4	0.48	0.19	129.51	0.16	0.08	12.55	85.52	99.02
5	2.93	0.75	144.87	0.34	7.45	13.47	93.19	95.50
6	0.07	0.46	109.90	0.19	0.08	12.22	58.90	65.31
7	0.15	0.25	123.47	0.19	0.46	11.70	82.16	94.05
8	0.12	1.53	108.03	0.38	0.22	11.45	82.90	96.88
9	0.16	0.22	98.03	0.13	1.25	10.84	62.21	83.14
10	0.90	0.45	123.38	0.18	0.29	9.53	94.79	90.26
11	0.08	0.13	106.81	0.13	0.28	10.80	57.55	86.79
12	0.09	0.23	149.37	0.21	1.30	15.43	58.05	70.62
13	0.03	0.13	110.29	0.14	0.08	13.21	56.86	106.44
14	0.09	0.13	153.70	0.18	0.16	14.44	68.11	123.03
15	0.14	0.46	118.79	0.26	0.09	14.84	73.39	154.56
16	0.09	0.55	116.72	0.28	3.72	13.18	57.23	131.05
17	0.14	1.00	126.81	0.24	1.19	21.51	113.62	156.26
18	0.13	0.14	189.11	0.20	0.19	16.69	106.08	234.80
19	0.23	0.35	120.61	0.34	7.50	16.18	65.31	138.42
20	0.08	0.29	109.47	0.27	2.16	12.91	50.47	87.28
21	0.66	1.08	99.38	0.13	0.99	16.41	64.51	107.07
22	0.07	0.19	105.54	0.23	2.03	11.98	59.43	106.48
23	0.11	0.14	122.83	0.16	1.75	17.55	57.86	90.44
24	0.32	0.13	118.95	0.25	1.60	19.18	396.21	167.27
25	0.24	0.15	176.53	0.60	0.38	12.61	215.55	140.54
26	0.09	0.17	210.55	0.48	0.30	12.48	92.53	122.03
27	0.11	0.29	194.20	0.47	0.37	11.74	121.39	201.55
28	0.10	0.13	170.02	0.62	0.08	18.00	73.23	100.37
29	0.32	0.32	132.56	0.29	0.17	15.00	212.99	266.52
30	0.36	0.13	191.27	0.38	0.84	13.72	361.59	272.05
31	0.11	0.18	109.47	0.26	2.74	15.23	83.59	161.15
32	0.05	0.21	99.54	0.36	2.32	19.64	44.46	136.36
33	0.10	0.52	156.69	0.11	1.02	11.30	84.05	128.96
34	0.76	0.13	140.31	0.59	3.63	19.32	57.58	107.70
35	0.58	0.13	94.47	0.14	1.74	15.52	88.91	163.23
mean	0.30	0.34	132.47	0.27	1.34	14.39	98.98	125.84
min	0.03	0.13	87.91	0.11	0.08	9.53	44.46	62.81
max	2.93	1.53	210.55	0.62	7.50	21.51	396.21	272.05
s.d.	0.50	0.31	32.15	0.14	1.84	2.90	79.59	52.47
first quartile	0.09	0.14	109.47	0.17	0.17	12.10	57.95	90.35
third quartile	0.32	0.40	151.54	0.34	1.74	16.29	92.86	147.55
median	0.14	0.22	122.83	0.23	0.46	13.72	73.39	107.07
LOD	0.027	0.131	0.070	0.033	0.082	0.080	0.021	0.026

3.2. Heavy metals in PM_{10} samples and relation with honey bee bioaccumulation

In the case of PM_{10} the results have been obtained from the background stations PGC, TRC, and MM, therefore are intended to represent the average air quality in the areas around the background stations. The average PM_{10} values (2013–2016) were 31.0, 23.9, and 9.9 Mg m^{-3} at TRC, PGC, and MM stations, respectively. The average values of all heavy metals in PM_{10} at three stations showed

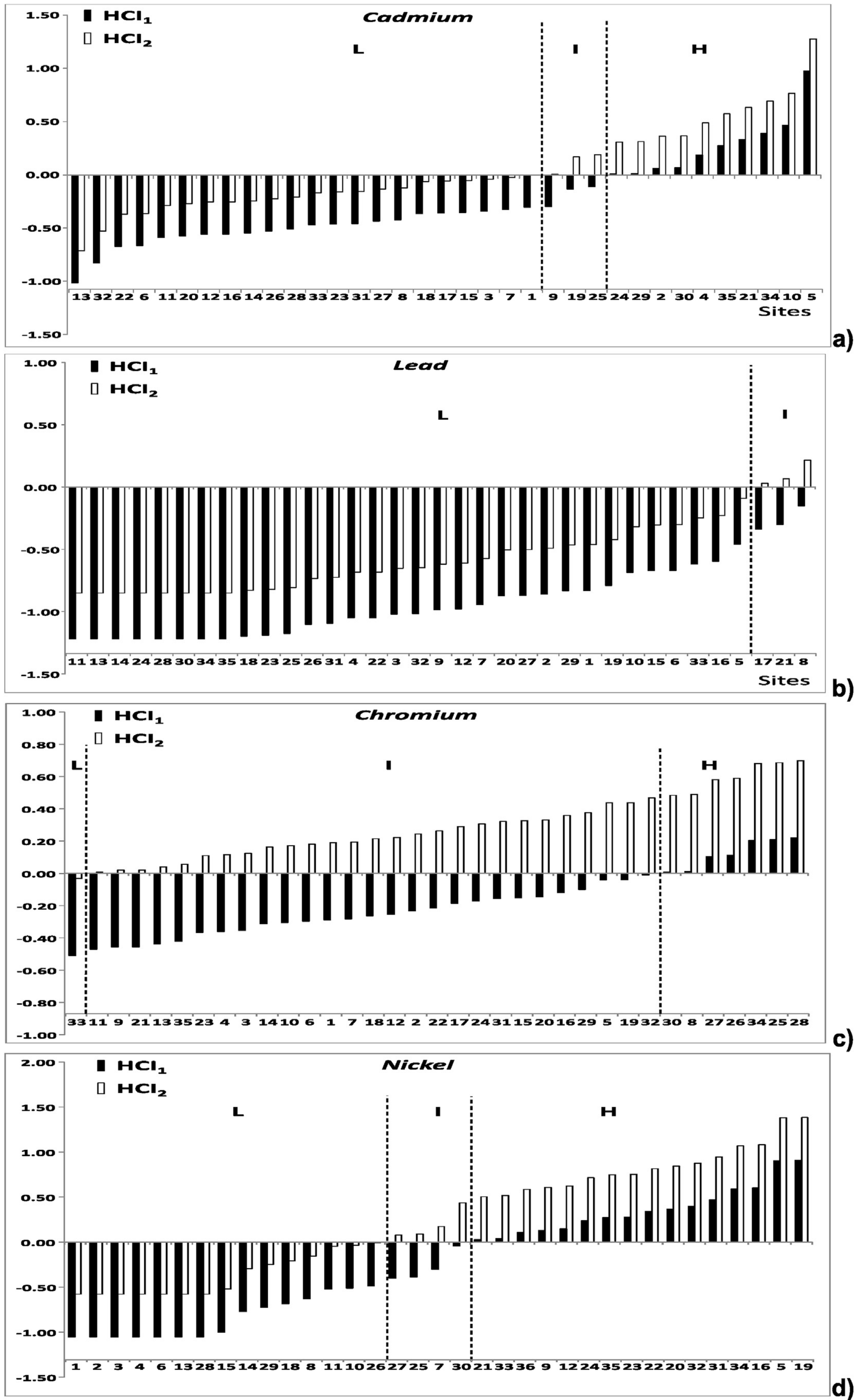

Fig. 2. a-d. HCI_1 and HCI_2 values for Cd (a), Pb (b), Cr (c) and Ni (d) in the 35 sampling sites - Umbria Region, Italy. HCI_1, black histogram; HCI_2, white histogram. *H, I,* and *L letters* represent HCI values that indicate high ($HCI_1 > 0$), intermediate ($HCI_1 < 0$ and $HCI_2 > 0$) and low ($HCI_2 < 0$) contamination condition, respectively.

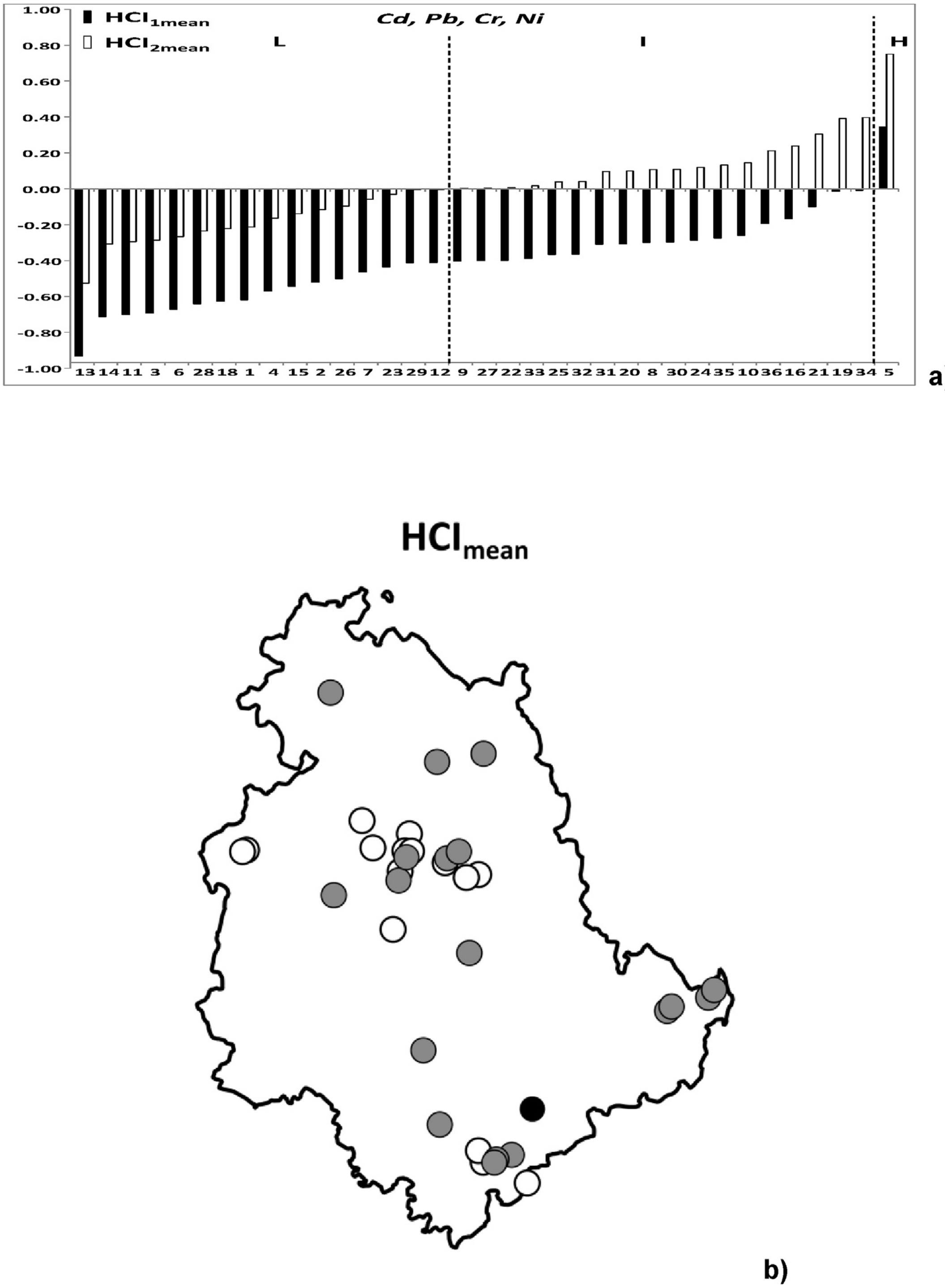

Fig. 3. a-b. HCI$_{mean}$ values (Cd, Pb, Cr, and Ni) of the 35 sampling sites - Umbria Region, Italy. (a) HCI$_{1mean}$, black histogram; HCI$_{2mean}$, white histogram; *H, I,* and *L letters* represent HCI$_{mean}$ values that indicate high (HCI$_{1mean}$ > 0), intermediate (HCI$_{1mean}$ < 0 and HCI$_{2mean}$ > 0) and low (HCI$_{2mean}$ < 0) contamination condition, respectively; (b) *black*, *grey* and *white spots* indicate high, intermediate and low HCI$_{mean}$ contamination condition, respectively.

significant differences between TRC *vs* PGC and MM (ANOVA and *post hoc* Tukey test, Table 2).

In the case of honey bees, in order to compute the PM_{10}, we averaged the metal concentrations of all the apiaries within the areas around PM_{10} background stations. Three areas have been individuated: *(i)* Perugino (sites: 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 13, 14, 16, and 18); *(ii)* Ternano (sites: 5, 24, 25, 26, 27, and 28); *(iii)* mountain (sites: 29, 34, and 35). The latter includes all the sites with elevation above 900 m a.s.l. therefore with characteristics similar to those of the MM site (Fig. 1, Table S1). Only the average values of Cr, Zn, and Mn in honey bees at three areas showed significant differences between Ternano and Perugino areas (ANOVA and *post hoc* Tukey test, Table 3).

Table 2
PM_{10} heavy metal concentrations (ng m^{-3}) in stations PGC, TRC and MM: mean, standard deviation (s.d.), letter (significant differences in *post hoc* Tukey test with a ¼ 0.05).

Stations		Cd	Pb	Cr	Ni	Zn	Cu	Mn	Fe
MM	mean	0.03^{b}	1.21^{b}	6.54^{b}	2.31^{b}	19.21^{a,b}	5.00^{b}	2.73^{b}	120.11^{b}
(2013e2016)	s.d.	0.03	0.96	7.21	2.83	19.20	5.44	2.75	172.90
PGC	mean	0.02^{b}	2.34^{b}	2.09^{b}	1.02^{b}	17.78^{b}	18.77^{a}	3.46^{b}	141.66^{b}
(2013e2014)	s.d.	0.01	2.21	1.75	1.12	19.71	13.70	3.58	157.12
TRC	mean	0.21^{a}	6.02^{a}	58.98^{a}	20.47^{a}	32.21^{a}	21.26^{a}	16.56^{a}	627.14^{a}
(2015e2016)	s.d.	0.24	4.42	49.48	16.65	30.68	13.57	11.08	389.83

Table 3
Honey bee heavy metal concentrations (dry weight, mg kg^{-1}) in Ternano, Perugino and mountain areas: mean, standard deviation (s.d.), letter (significant differences in *post hoc* Tukey test with a ¼ 0.05).

Areas		Cd	Pb	Cr	Ni	Zn	Cu	Mn	Fe
Mountain	mean	0.55^{a}	0.19^{a}	0.34^{a,b}	1.85^{a}	122.44^{a,b}	16.61^{a}	119.83^{a,b}	179.15^{b}
	s.d.	0.22	0.11	0.23	1.73	24.54	2.35	82.19	80.60
Perugino	mean	0.21^{a}	0.36^{a}	0.20^{b}	0.50^{a}	124.58^{b}	12.83^{a}	71.16^{b}	102.14^{a}
	s.d.	0.23	0.36	0.07	0.98	28.31	2.15	16.79	42.78
Ternano	mean	0.63^{a}	0.27^{a}	0.46^{a}	1.70^{a}	169.18^{a}	14.58^{a}	165.35^{a}	137.88^{a,b}
	s.d.	1.13	0.24	0.14	2.87	33.19	3.18	123.89	40.93

4. Discussion

Studies on contamination of terrestrial environments are more complex than those on freshwater environments where the habitat is defined and limited within precise dimensions (Pallottini et al., 2017a). However, efficient bioindicators allow a proper evaluation of the environmental quality in terrestrial environments contaminated by pollutants (Devillers and Pham-Delègue, 2002; Goretti et al., 2011). In our study, we used honey bees as bioindicators and we measured the heavy metal bioaccumulation extent in the honey bee matrix of 35 sites distributed in the Umbria Region territory.

4.1. Heavy metal levels in honey bees: comparison with Italian studies

The first objective of the present work was the comparison of the results with other studies from Italy, in particular from Central Italy, allowing the evaluation of the heavy metal honey bee bioaccumulation in a national context.

Leita et al. (1996) at one site (near an extra-urban crossroad; monitored weekly for 9 weeks), reported mean values (mg kg^{-1} d.w.) for Cd of 1.37, Pb of 1.82, and Zn of 61.04, respectively 4.6, 5.3, and 0.5 folds the mean values of the present study.

Conti and Botrè (2001) at five sites from Rome and Province (of which A, D, and E sites are considered not polluted; B is relatively close to a highway and C is moderately polluted and located in an area with intense motor vehicles circulation; monitored in 2 periods, 4e6/12 weeks) reported mean values (mg kg^{-1} d.w.) for Cd of 3.27, Pb of 0.83, and Cr of 0.07, respectively 10.9, 2.4, and 0.3 folds the mean values of the present study.

Perugini et al. (2011) at eight sites from Lazio and Abruzzi (of which sites 2, 3, 4, and 5 are unpolluted inside wildlife reserves; 6 and 7 are inside a reserve but not too far from the main thoroughfare; 8 is close to an airport with intense air traffic and motor vehicle circulation; site 1 is placed in a moderately polluted area, near a small road and an incinerator) reported mean values (mg kg^{-1} d.w.) for Cd of 0.05, Pb of 0.38, and Cr of 0.74, respectively 0.2, 1.1, and 2.7 folds the mean values of the present study.

Ruschioni et al. (2013) at eleven sites from Natural Reserves of Marche Region (of which sites C, D, F, G, I, and K are considered wilder areas; A, B, and E are surrounded by agricultural environments; H and J are surrounded by industrial and urban environments; monitored in 2008e2010) reported mean values (mg kg^{-1} d.w.) for Cd of 0.06, Pb of 0.22, Cr of 0.11, and Ni of 0.12 that are respectively 0.2, 0.6, 0.4, and 0.1 folds the mean values of the present study.

Giglio et al. (2017) at two sites from Trieste city (urban, located in a city garden and suburban, located close to an industrial area) reported mean values (mg kg^{-1} d.w.) for Cd of 0.04, Pb of 0.12, Cr of 0.36, Ni of 0.39, Zn of 52.02, and Cu of 15.38 respectively 0.1, 0.4, 1.3, 0.3, 0.4 and 1.1 folds the mean values of the present study.

In general, the comparison of the data collected in our study with selected Italian studies showed in Umbria an average bioaccumulation value of heavy metals in honey bee matrix of the same order of magnitude, with Cd that showed a higher variability (see Table S2 in Supplementary Material).

4.2. Application of the honey bee contamination index: from regional to European context

A second goal of the present study aimed at outlining a picture of the heavy metal contamination levels employing a contamination index. An index should evaluate the effects of pollution on the biotic community (Fabrizi et al., 2010; Mondy et al., 2012; Dedieu et al., 2016. Pallottini et al., 2017b) or measure the contamination level of toxicants on target species (Nieminen et al., 2001; Goretti et al., 2016, 2018). Honeybee Contamination Index (HCI) belongs to the latter category and evaluates heavy metal contamination in terrestrial environments through the use of *Apis mellifera* as bioindicator.

The HCI application to our data (Umbria) gave evidence of a high contamination level (mainly of Cd and Ni) present at site 5 (placed between Terni and Spoleto, Fig. 1) that has been probably influenced by the Terni's steel plant located close to the apiary site (about 5 km), whose emissions, crossing a narrow valley, reach the territory explored by the forager bees. Therefore, we may hypothesize, for this specific site, a more direct relationship with airborne heavy metals of industrial provenance. In contrast, sites 28 and 26, located in the Terni Valley but in the opposite side from site 5, showed a low contamination level.

The Umbrian site with the lowest contamination condition was site 13. This site, together with other nine sites characterized by low heavy metal contamination, outlined a territory portion (within Perugino area) between Tezio and Malbe Mountains and Subasio

Table 4
HCI_{1mean} and HCI_{2mean} values (in ascending order) of the present study (P.S., *in grey*) in relation to Italian metedata, in particular from Central Italy. *Ref. 1*, Ruschioni et al. (2013); *Ref. 2*, Perugini et al. (2011); *Ref. 3*, Conti & Botrè (2001); *Ref. 4*, Giglio et al. (2017); *Ref. 5*, Leita et al. (1996). For each reference the site codes are indicated. *H, I,* and *L letters* represent HCI_{mean} values in high, intermediate and low contamination condition, respectively.

Ref.	Site (cod.)	Heavy metals	Metals (N.)	HCI_{1mean}	HCI_{2mean}	HCI_{mean}
P.S.	13	Cd, Pb, Cr, Ni	4	−0.93	−0.53	L
P.S.	14	Cd, Pb, Cr, Ni	4	−0.71	−0.31	L
P.S.	11	Cd, Pb, Cr, Ni	4	−0.70	−0.29	L
P.S.	3	Cd, Pb, Cr, Ni	4	−0.69	−0.29	L
P.S.	6	Cd, Pb, Cr, Ni	4	−0.67	−0.27	L
Ref. 1	H_10	Cd, Pb, Cr, Ni	4	−0.67	−0.26	L
Ref. 4	urban	Cd, Pb, Cr, Ni	4	−0.66	−0.25	L
Ref.4	suburban	Cd, Pb, Cr, Ni	4	−0.64	−0.24	L
P.S.	28	Cd, Pb, Cr, Ni	4	−0.64	−0.23	L
P.S.	18	Cd, Pb, Cr, Ni	4	−0.63	−0.22	L
P.S.	1	Cd, Pb, Cr, Ni	4	−0.62	−0.21	L
Ref. 1	J_10	Cd, Pb, Cr, Ni	4	−0.60	−0.20	L
P.S.	4	Cd, Pb, Cr, Ni	4	−0.57	−0.16	L
Ref. 1	G_10	Cd, Pb, Cr, Ni	4	−0.56	−0.15	L
P.S.	15	Cd, Pb, Cr, Ni	4	−0.54	−0.14	L
Ref. 1	E_10	Cd, Pb, Cr, Ni	4	−0.53	−0.13	L
Ref. 1	D_09	Cd, Pb, Cr, Ni	4	−0.53	−0.12	L
Ref. 2	2	Cd, Pb, Cr	3	−0.52	−0.14	L
P.S.	2	Cd, Pb, Cr, Ni	4	−0.52	−0.12	L
P.S.	26	Cd, Pb, Cr, Ni	4	−0.50	−0.10	L
Ref. 1	K_09	Cd, Pb, Cr, Ni	4	−0.49	−0.09	L
Ref. 2	3	Cd, Pb, Cr	3	−0.48	−0.10	L
Ref. 1	B_09	Cd, Pb, Cr, Ni	4	−0.47	−0.06	L
P.S.	7	Cd, Pb, Cr, Ni	4	−0.46	−0.06	L
Ref. 1	I_10	Cd, Pb, Cr, Ni	4	−0.45	−0.05	L
Ref. 1	H_08	Cd, Pb, Cr, Ni	4	−0.45	−0.04	L
Ref. 1	B_10	Cd, Pb, Cr, Ni	4	−0.45	−0.04	L
Ref. 1	K_10	Cd, Pb, Cr, Ni	4	−0.44	−0.04	L
Ref. 2	1	Cd, Pb, Cr	3	−0.44	−0.05	L
P.S.	23	Cd, Pb, Cr, Ni	4	−0.44	−0.03	L
Ref. 1	J_09	Cd, Pb, Cr, Ni	4	−0.42	−0.02	L
Ref. 1	G_09	Cd, Pb, Cr, Ni	4	−0.42	−0.02	L
Ref. 2	6	Cd, Pb, Cr	3	−0.42	−0.04	L
P.S.	29	Cd, Pb, Cr, Ni	4	−0.41	−0.01	L
Ref. 1	B_08	Cd, Pb, Cr, Ni	4	−0.41	−0.01	L
P.S.	12	Cd, Pb, Cr, Ni	4	−0.41	−0.01	L
Ref. 2	4	Cd, Pb, Cr	3	−0.41	−0.03	L
P.S.	9	Cd, Pb, Cr, Ni	4	−0.40	0.00	I
P.S.	27	Cd, Pb, Cr, Ni	4	−0.40	0.01	I
P.S.	22	Cd, Pb, Cr, Ni	4	−0.40	0.01	I
P.S.	33	Cd, Pb, Cr, Ni	4	−0.39	0.02	I
Ref. 2	7	Cd, Pb, Cr	3	−0.39	−0.01	L
Ref. 1	A_10	Cd, Pb, Cr, Ni	4	−0.37	0.04	I
Ref. 2	8	Cd, Pb, Cr	3	−0.37	0.01	I
P.S.	25	Cd, Pb, Cr, Ni	4	−0.37	0.04	I
Ref. 2	5	Cd, Pb, Cr	3	−0.37	0.02	I
P.S.	32	Cd, Pb, Cr, Ni	4	−0.36	0.04	I
Ref. 1	H_09	Cd, Pb, Cr, Ni	4	−0.34	0.06	I
Ref. 1	D_08	Cd, Pb, Cr, Ni	4	−0.34	0.07	I
Ref. 1	F_08	Cd, Pb, Cr, Ni	4	−0.33	0.08	I
Ref. 1	D_10	Cd, Pb, Cr, Ni	4	−0.33	0.08	I
Ref. 1	C_09	Cd, Pb, Cr, Ni	4	−0.32	0.08	I
Ref. 1	F_10	Cd, Pb, Cr, Ni	4	−0.31	0.10	I
P.S.	31	Cd, Pb, Cr, Ni	4	−0.31	0.10	I
P.S.	20	Cd, Pb, Cr, Ni	4	−0.31	0.10	I
P.S.	8	Cd, Pb, Cr, Ni	4	−0.30	0.11	I
P.S.	30	Cd, Pb, Cr, Ni	4	−0.30	0.11	I
Ref. 1	J_08	Cd, Pb, Cr, Ni	4	−0.29	0.12	I
Ref. 1	G_08	Cd, Pb, Cr, Ni	4	−0.29	0.12	I
Ref. 1	I_09	Cd, Pb, Cr, Ni	4	−0.29	0.12	I
P.S.	24	Cd, Pb, Cr, Ni	4	−0.29	0.12	I
P.S.	35	Cd, Pb, Cr, Ni	4	−0.27	0.13	I
P.S.	10	Cd, Pb, Cr, Ni	4	−0.26	0.15	I
Ref. 1	F_09	Cd, Pb, Cr, Ni	4	−0.25	0.15	I
Ref. 1	A_09	Cd, Pb, Cr, Ni	4	−0.24	0.17	I
Ref. 1	E_08	Cd, Pb, Cr, Ni	4	−0.23	0.18	I
Ref. 1	E_09	Cd, Pb, Cr, Ni	4	−0.22	0.19	I
Ref. 1	C_08	Cd, Pb, Cr, Ni	4	−0.22	0.19	I
Ref. 1	K_08	Cd, Pb, Cr, Ni	4	−0.21	0.20	I
Ref. 1	C_10	Cd, Pb, Cr, Ni	4	−0.20	0.20	I
Ref. 1	A_08	Cd, Pb, Cr, Ni	4	−0.20	0.21	I
P.S.	17	Cd, Pb, Cr, Ni	4	−0.19	0.21	I
P.S.	16	Cd, Pb, Cr, Ni	4	−0.17	0.24	I
Ref. 3	B2	Cd, Pb, Cr	3	−0.12	0.26	I
Ref. 3	B1	Cd, Pb, Cr	3	−0.12	0.27	I
Ref. 3	A1	Cd, Pb, Cr	3	−0.11	0.27	I
P.S.	21	Cd, Pb, Cr, Ni	4	−0.10	0.31	I
Ref. 3	A2	Cd, Pb, Cr	3	−0.08	0.30	I
Ref. 3	E1	Cd, Pb, Cr	3	−0.07	0.31	I
Ref. 3	D2	Cd, Pb, Cr	3	−0.07	0.31	I
Ref. 1	I_08	Cd, Pb, Cr, Ni	4	−0.07	0.34	I
Ref. 3	D1	Cd, Pb, Cr	3	−0.06	0.32	I
Ref. 3	E2	Cd, Pb, Cr	3	−0.05	0.33	I
P.S.	19	Cd, Pb, Cr, Ni	4	−0.01	0.39	I
P.S.	34	Cd, Pb, Cr, Ni	4	−0.01	0.40	I
Ref. 3	C2	Cd, Pb, Cr	3	0.10	0.48	H
Ref. 3	C1	Cd, Pb, Cr	3	0.12	0.50	H
Ref. 5	week1	Cd, Pb	2	0.23	0.56	H
Ref. 5	week3	Cd, Pb	2	0.23	0.57	H
Ref. 5	week2	Cd, Pb	2	0.25	0.58	H
Ref. 5	week7	Cd, Pb	2	0.25	0.59	H
Ref. 5	week8	Cd, Pb	2	0.26	0.59	H
Ref. 5	week4	Cd, Pb	2	0.26	0.59	H
Ref. 5	week5	Cd, Pb	2	0.27	0.60	H
Ref. 5	week6	Cd, Pb	2	0.31	0.65	H
P.S.	5	Cd, Pb, Cr, Ni	4	0.34	0.75	H
Ref. 5	week9	Cd, Pb	2	0.40	0.73	H

Mountain with good environmental quality (Fig. 1).

A comparison of HCI values at Italian level has been possible through the analysis of the following studies carried out in Italy: *(i)* Cd, Pb, Ni, and Cr in Ruschioni et al. (2013) and Giglio et al. (2017), *(ii)* Cd, Pb, and Cr in Conti and Botrè (2001), Perugini et al. (2011), *(iii)* Cd and Pb in Leita et al. (1996). The HCI application to the metadata for Italy showed a wide range of 62 HCI values, of which 22, 29 and 11 values are of low, intermediate and high contamination level, respectively.

The comparison of Italian metadata with the 35 HCI values from our study highlighted that most of the Umbrian sites fell in the portion of low and intermediate contamination condition of this Italian series. However, it is interesting to note that sites 13, 14, 11, 3, and 6 (within Perugino area) displayed the lowest contamination of the HCI Italian series, while site 5 (within the Ternano area) was among the sites with the highest contamination of this series (Table 4). Besides, the comparison of HCI trends for individual metals between our study and the Italian metadata are shown in Table S3 of the Supplementary Material.

An extended comparison at European level has been possible through the analysis of the surveys carried out on the heavy metals bioaccumulation in honey bees from various authors, in particular for: *(i)* Cd and Pb in Finland (Fakhimzadeh and Lodenius, 2000) at twelve sites (F*, H*, I*, K* control sites; A(i), B(i), D(i), J(i) placed in industrial areas; C(u), E(u), G(u), L(u) located in urban areas); *(ii)* Pb, Cd, Cr, and Ni in Netherland (Van der Steen et al., 2012) at three sites (site Ma, placed in an urban area with industries; site Bu, in a rural area with a power plant; site Ho, close to a port and a large industrial area; monitored in July, August and September); *(iii)* Cd, Pb, Cr and Ni in Spain (Gutiérrez et al., 2015) at five sites (S1, placed in agricultural area close to a river; S2 and S3 control sites; S4, located in urban area; S5, placed in an abandoned quarry); *(iv)* Cd and Pb in Poland (Roman, 2010) at fourteen sites (1e7 apiaries placed in urban region; 8e14 apiaries in agricultural-woodland region); *(v)* Cd, Cr and Ni in Serbia (Zarić et al., 2017) at two locations (PA, Pančevo city with an industrial background and VS, Vršac

Table 5

HCI_{1mean} and HCI_{2mean} values (in ascending order) of the present study (P.S., *in grey*) in relation to metedata from various European regions. *Ref. 1*, Fakhimzadeh & Lodenius (2000); *Ref. 2*, Van der Steen et al. (2012); *Ref. 3*, Gutiérrez et al. (2015); *Ref. 4*, Roman (2010); *Ref. 5*, Zarić et al. (2017); *Ref. 6*, Zarić et al. (2016); *Ref. 7*, Van der Steen et al. (2016). For each reference the site codes are indicated. *H, I,* and *L letters* represent HCI_{mean} values in high, intermediate and low contamination condition, respectively.

Reference	Site (cod.)	Heavy metals	Metals (N.)	HCI_{1mean}	HCI_{2mean}	HCI_{mean}
P.S.	13	Cd, Pb, Cr, Ni	4	−0.93	−0.53	L
Ref. 1	K *	Cd, Pb	2	−0.78	−0.44	L
Ref. 5	PA_15	Cd, Cr, Ni	3	−0.73	−0.31	L
Ref. 1	J (i)	Cd, Pb	2	−0.72	−0.39	L
P.S.	14	Cd, Pb, Cr, Ni	4	−0.71	−0.31	L
P.S.	11	Cd, Pb, Cr, Ni	4	−0.70	−0.29	L
Ref. 1	L (u)	Cd, Pb	2	−0.70	−0.36	L
P.S.	3	Cd, Pb, Cr, Ni	4	−0.69	−0.29	L
Ref. 1	H *	Cd, Pb	2	−0.68	−0.35	L
P.S.	6	Cd, Pb, Cr, Ni	4	−0.67	−0.27	L
P.S.	28	Cd, Pb, Cr, Ni	4	−0.64	−0.23	L
Ref. 2	Hoek3	Cd, Pb, Cr, Ni	4	−0.63	−0.22	L
Ref. 2	Bugg3	Cd, Pb, Cr, Ni	4	−0.63	−0.22	L
P.S.	18	Cd, Pb, Cr, Ni	4	−0.63	−0.22	L
P.S.	1	Cd, Pb, Cr, Ni	4	−0.62	−0.21	L
Ref. 2	Hoek4	Cd, Pb, Cr, Ni	4	−0.61	−0.21	L
Ref. 5	VS_14	Cd, Cr, Ni	3	−0.61	−0.19	L
Ref. 2	Maas3	Cd, Pb, Cr, Ni	4	−0.59	−0.19	L
P.S.	4	Cd, Pb, Cr, Ni	4	−0.57	−0.16	L
Ref. 2	Hoek2	Cd, Pb, Cr, Ni	4	−0.56	−0.15	L
Ref. 1	D (i)	Cd, Pb	2	−0.55	−0.22	L
P.S.	15	Cd, Pb, Cr, Ni	4	−0.54	−0.14	L
Ref. 1	C (u)	Cd, Pb	2	−0.54	−0.20	L
P.S.	2	Cd, Pb, Cr, Ni	4	−0.52	−0.12	L
P.S.	26	Cd, Pb, Cr, Ni	4	−0.50	−0.10	L
Ref. 2	Maas4	Cd, Pb, Cr, Ni	4	−0.49	−0.09	L
Ref. 2	Bugg1	Cd, Pb, Cr, Ni	4	−0.49	−0.09	L
Ref. 1	F *	Cd, Pb	2	−0.47	−0.14	L
Ref. 2	Maas2	Cd, Pb, Cr, Ni	4	−0.47	−0.06	L
Ref. 2	Bugg4	Cd, Pb, Cr, Ni	4	−0.47	−0.06	L
P.S.	7	Cd, Pb, Cr, Ni	4	−0.46	−0.06	L
Ref. 2	Maas1	Cd, Pb, Cr, Ni	4	−0.45	−0.04	L
P.S.	23	Cd, Pb, Cr, Ni	4	−0.44	−0.03	L
Ref. 3	S2_09	Pb, Cr, Ni	3	−0.41	0.03	I
P.S.	29	Cd, Pb, Cr, Ni	4	−0.41	−0.01	L
P.S.	12	Cd, Pb, Cr, Ni	4	−0.41	−0.01	L
Ref. 3	S3_07	Pb, Cr, Ni	3	−0.41	0.03	I
Ref. 6	MS	Cd, Pb, Cr, Ni	4	−0.41	0.00	I
P.S.	9	Cd, Pb, Cr, Ni	4	−0.40	0.00	I
P.S.	27	Cd, Pb, Cr, Ni	4	−0.40	0.01	I
P.S.	22	Cd, Pb, Cr, Ni	4	−0.40	0.01	I
Ref. 2	Hoek6	Cd, Pb, Cr, Ni	4	−0.40	0.01	I
Ref. 3	S5_09	Pb, Cr, Ni	3	−0.39	0.05	I
Ref. 6	BG	Cd, Pb, Cr, Ni	4	−0.39	0.02	I
Ref. 1	I *	Cd, Pb	2	−0.39	−0.06	L
P.S.	33	Cd, Pb, Cr, Ni	4	−0.39	0.02	I
Ref. 2	Maas5	Cd, Pb, Cr, Ni	4	−0.38	0.02	I
Ref. 2	Bugg5	Cd, Pb, Cr, Ni	4	−0.38	0.03	I
Ref. 1	G (u)	Cd, Pb	2	−0.37	−0.04	L
P.S.	25	Cd, Pb, Cr, Ni	4	−0.37	0.04	I
P.S.	32	Cd, Pb, Cr, Ni	4	−0.36	0.04	I
Ref. 3	S3_09	Pb, Cr, Ni	3	−0.36	0.08	I
Ref. 3	S2_10	Cd, Pb, Cr, Ni	4	−0.35	0.05	I
Ref. 3	S1_10	Cd, Pb, Cr, Ni	4	−0.34	0.06	I
Ref. 1	E (u)	Cd, Pb	2	−0.33	0.00	I
Ref. 5	PA_14	Cd, Cr, Ni	3	−0.33	0.09	I
Ref. 3	S1_09	Pb, Cr, Ni	3	−0.33	0.11	I
Ref. 3	S5_10	Cd, Pb, Cr, Ni	4	−0.32	0.08	I
Ref. 5	VS_15	Cd, Cr, Ni	3	−0.32	0.10	I
Ref. 1	A (i)	Cd, Pb	2	−0.32	0.02	I
Ref. 3	S4_10	Cd, Pb, Cr, Ni	4	−0.31	0.09	I
Ref. 3	S3_10	Cd, Pb, Cr, Ni	4	−0.31	0.09	I
P.S.	31	Cd, Pb, Cr, Ni	4	−0.31	0.10	I
P.S.	20	Cd, Pb, Cr, Ni	4	−0.31	0.10	I
Ref. 6	TPP	Cd, Pb, Cr, Ni	4	−0.30	0.10	I
P.S.	8	Cd, Pb, Cr, Ni	4	−0.30	0.11	I
P.S.	30	Cd, Pb, Cr, Ni	4	−0.30	0.11	I
Ref. 2	Hoek1	Cd, Pb, Cr, Ni	4	−0.30	0.11	I
P.S.	24	Cd, Pb, Cr, Ni	4	−0.29	0.12	I
P.S.	35	Cd, Pb, Cr, Ni	4	−0.27	0.13	I
P.S.	10	Cd, Pb, Cr, Ni	4	−0.26	0.15	I
Ref. 3	S4_09	Pb, Cr, Ni	3	−0.25	0.19	I
Ref. 2	Bugg2	Cd, Pb, Cr, Ni	4	−0.22	0.18	I
Ref. 2	Bugg6	Cd, Pb, Cr, Ni	4	−0.21	0.20	I
P.S.	17	Cd, Pb, Cr, Ni	4	−0.19	0.21	I
Ref. 2	Maas6	Cd, Pb, Cr, Ni	4	−0.19	0.22	I
P.S.	16	Cd, Pb, Cr, Ni	4	−0.17	0.24	I
Ref. 3	S5_07	Pb, Cr, Ni	3	−0.16	0.28	I
Ref. 7	16 > Urban	Cd, Cr, Ni	3	−0.14	0.27	I
Ref. 3	S1_07	Pb, Cr, Ni	3	−0.14	0.30	I
P.S.	21	Cd, Pb, Cr, Ni	4	−0.10	0.31	I
Ref. 2	Hoek5	Cd, Pb, Cr, Ni	4	−0.10	0.31	I
Ref. 7	94 > Agric.	Cd, Cr, Ni	3	−0.09	0.33	I
Ref. 7	30_Mixed	Cd, Cr, Ni	3	−0.06	0.36	I
Ref. 4	12	Cd, Pb	2	−0.03	0.31	I
P.S.	19	Cd, Pb, Cr, Ni	4	−0.01	0.39	I
P.S.	34	Cd, Pb, Cr, Ni	4	−0.01	0.40	I
Ref. 5	PA_13	Cd, Cr, Ni	3	−0,00	0.41	I
Ref. 3	S4_07	Pb, Cr, Ni	3	0.01	0.46	H
Ref. 4	13	Cd, Pb	2	0.04	0.37	H
Ref. 4	5	Cd, Pb	2	0.05	0.39	H
Ref. 1	B (i)	Cd, Pb	2	0.05	0.39	H
Ref. 4	7	Cd, Pb	2	0.05	0.39	H
Ref. 4	8	Cd, Pb	2	0.08	0.41	H
Ref. 5	VS_13	Cd, Cr, Ni	3	0.09	0.51	H
Ref. 4	1	Cd, Pb	2	0.09	0.42	H
Ref. 4	3	Cd, Pb	2	0.11	0.44	H
Ref. 4	14	Cd, Pb	2	0.12	0.45	H
Ref. 7	7 > Wood	Cd, Cr, Ni	3	0.12	0.54	H
Ref. 4	4	Cd, Pb	2	0.14	0.47	H
Ref. 4	9	Cd, Pb	2	0.14	0.48	H
Ref. 4	6	Cd, Pb	2	0.16	0.50	H
Ref. 4	10	Cd, Pb	2	0.18	0.51	H
Ref. 4	2	Cd, Pb	2	0.21	0.54	H
Ref. 4	11	Cd, Pb	2	0.31	0.65	H
P.S.	5	Cd, Pb, Cr, Ni	4	0.34	0.75	H

city surrounded by an agricultural landscape, monitored in 2013, 2014 and 2015); *(vi)* Cd, Pb, Cr and Ni in Serbia (Zarić et al., 2016) at three locations (TPP, located around two thermal power plants of Kostolac city; BG, Belgrade city; MS, rural region of Mesić village); *(vii)* Cd, Cr and Ni in Netherland (Van der Steen et al., 2016) at 147 sites, grouped in four categories according to land use (94 sites with >50% agricultural area; 7 sites with >50% woods; 16 sites with >50% urban area; 30 sites with mixed land use).

The HCI application to the metadata for Europe showed a wide range of 71 HCI values, of which 21, 33 and 17 values are of low, intermediate and high contamination level, respectively.

The HCI application to European metadata and the comparison with the 35 HCI values of our study showed, even in this context, that most of the Umbrian sites fell in the portion of low and intermediate contamination condition of this European series. It is important to note that the lowest (site 13) and the highest (site 5) of the HCI values of this series of comparison data belonged to the Umbria territory (Table 5).

4.3. *Sources and mechanisms of metal bioaccumulation*

The final objective of the present work was to identify the sources and mechanisms of heavy metals uptake (bioaccumulation) by the honey bees. Airborne heavy metals are present in the atmosphere in coarse (diameters larger than 1 μm) and fine (diameters smaller than 1 μm) aerosol particles. Typical sources of fine aerosols are vehicular traffic and industrial activities while the

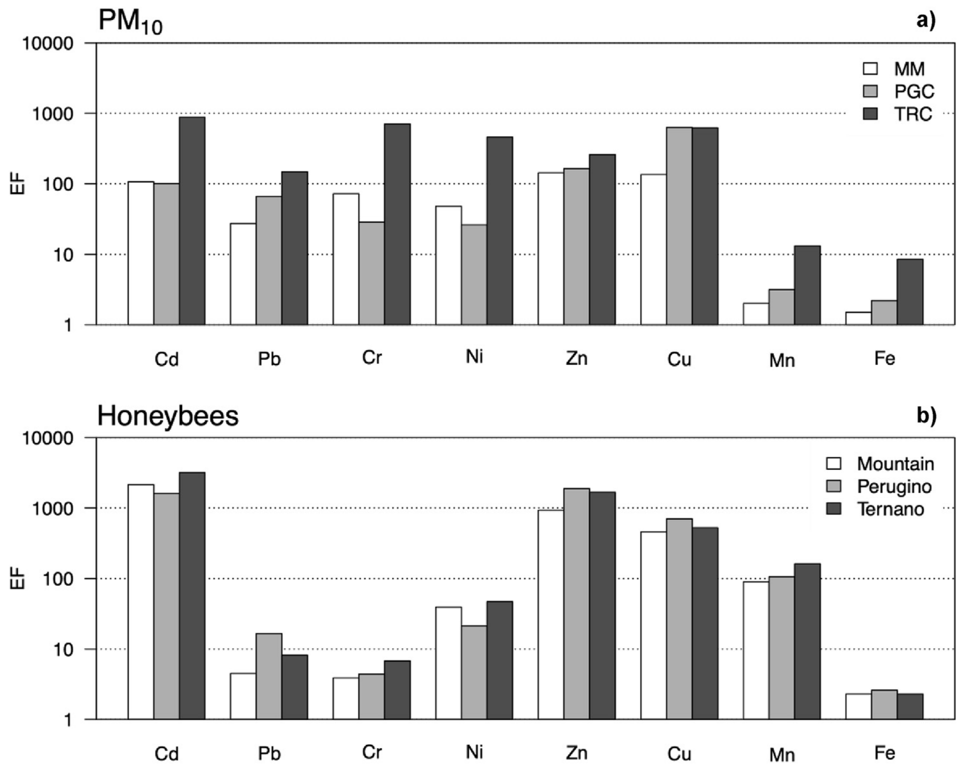

Fig. 4. a-b. Heavy metal Enrichment Factors (EF) for PM_{10} (a) and honey bee samples (b). (a): PGC, Perugia city; TRC, Terni city; MM, Martano Mountain. (b): Mountain (sites: 29, 34, and 35); Perugino (sites: 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 13, 14, 16, and 18); Ternano (sites: 5, 24, 25, 26, 27, and 28).

coarser fraction is usually generated by mechanical soil erosion and resuspension and contains elements typical of the Earth crust and soil contaminants. This work is based on the metal content of PM_{10} (particles diameters smaller than 10 μm) which includes both coarse and fine aerosols. If the soil is contaminated, for example an urban road or a fertilized agricultural soil, the resuspended particulate matter maybe enriched of heavy metals typical of traffic or fertilizers and pesticides. This resuspended fraction is coarse in size and has a shorter residence time in the atmosphere. For the above reasons, the metal content of PM_{10} represents the background concentrations in a relatively large area while the metal content of soil will affect the heavy metals concentrations only locally. Heavy metals may be uptaken by honey bees on vegetation where they are deposited through dry or wet processes or directly uptaken on-the-flight from locally resuspended particles. Therefore the metal content in honey bees should reflect mainly the local impact due to soil resuspension.

The main result of the comparison between PM_{10} and honey bees gave evidence that in PM_{10} all the metals showed a significantly larger concentrations in TRC with respect to PGC and MM stations, while in the honey bees only Cr, Zn, and Mn showed a similar significant difference between Ternano area and Perugino area, suggesting PM_{10} being a possible source only for these metals. However a comparison of the absolute concentrations could not be the best way to individuate a source in the case of airborne metals. Metal enrichment factors (Zhang et al., 2008) are better suited to this aim because the concentrations are scaled for a "natural" element ratio, usually that of the upper continental crust. The Enrichment factor (EF) of a heavy metal (X) can be calculated with the following standard relationship (see i.e. Gao et al., 1992; Arditsoglou and Samara, 2005; Cesari et al., 2012):

$$EF = [(C_X/C_{Al})\ sample] / [(C_X/C_{Al})\ upper\ continental\ crust]$$

where C_X and C_{Al} are the mean concentrations of X and of aluminium in different PM_{10} (or honey bee, see below) samples of the defined geographical areas. In the present work average elemental concentration data of the upper continental crust have been taken from Wedepohl (1995) (see Supplementary Material), although other choices are possible (see i.e. Cesari et al., 2012). Aluminium has been used as a reference element, assuming that its anthropogenic sources to the atmosphere are low (Gao et al., 1992). Operationally, given the local variation in soil composition and using the average upper crust composition as a reference, a value of EF > 20 suggests that a significant fraction of the element is contributed by non-crustal sources (Cesari et al., 2012). For EFs <10 crustal is the predominant source.

The EF results showed a higher contamination condition consistently by heavy metals in the particulate matter of TRC (Fig. 4a). The presence of elevated EF values in TRC aerosols has already been demonstrated in the past (Moroni et al., 2012) respect to PGC and MM aerosols. This evidence has been related to the presence of the intensive industrial activities in the Terni basin, in particular, those associated with stainless steel production.

Since recently, the accepted hypothesis has been that bees accumulate toxicants and can ultimately die by collecting pollen and nectar contaminated by atmospheric deposition, especially from residues of corn seeds treating (Pistorius et al., 2009). Moreover, Tapparo et al. (2012) demonstrated that bees approaching the drilling machine, while sowing, could also be contaminated on their flight by resuspended material from the drillings. These particles contained neonicotinoids, which have been demonstrated lethal to honey bees. Activities such as sowing and mechanical working of crop fields can resuspend in the local air not only neonicotinoids but also heavy metals. The metals in a crop field can be of natural origin, added by farmers through the use of fertilizers and pesticides or due to atmospheric deposition via atmospheric aerosols.

To exploit further hypotheses we tried an analysis of metal Enrichment Factors for honey bee samples (Fig. 4b). Shortly, the EF analysis in the honey bees did not show a gradient among the geographical areas as they did in PM_{10}. The high and relatively homogeneous EF values in the honey bees, in particular those of Cd, suggest the presence of another contamination source besides PM_{10}, which is most probably the ubiquitous use of phosphate fertilizers (Roberts, 2014) and pesticides (Gimeno-Garcia et al., 1996) containing metal contaminants. This local source may also be responsible for the patchy distribution of the HCI observed before (Fig. 3b).

5. Conclusion

The heavy metal bioaccumulation extent in honey bees allowed to outline a picture of the contamination level in the territory of Umbria Region (Italy).

The formulation of the Honeybee Contamination Index (HCI) provided an assessment of heavy metal contamination allowing to: *(i)* synthetically express the extent of heavy metal contamination measured in honey bee matrix; *(ii)* formulate a judgment of regional quality of terrestrial environments; *(iii)* compare the environmental quality among different territories.

Analysis of metal contamination in the airborne particulate matter (PM_{10}) and honey bees have been performed for specific areas under scrutiny on the present work. The results suggest the presence of another heavy metal contamination sources in honey bees besides PM_{10}, probably local activities, like sowing and mechanical working of crop fields, fertilization and use of pesticides. The effect of this local source resulted in an irregular geographical distribution of the environmental quality expressed by the HCI.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

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