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Article

Honeybees as Bioindicators of Heavy Metal Pollution in Urban and Rural Areas in the South of Italy

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Abstract: The honeybee (*Apis mellifera* L.) has been used in several studies for monitoring the environmental health status in terms of pollution, due to its wide-ranging foraging flights. Based on this consideration, this study aimed to analyze heavy metal pollution in Molise Region (Italy), by investigating five sites characterized by different levels of contamination. Furthermore, the authors carried out a sampling activity for a long period, in order to obtain a complete dataset. In this way, detailed information about the status of the environments was able to be obtained. The main purpose of this work was to assess the health status of Molise Region and to confirm the suitability of honeybees as environmental bioindicators of heavy metal pollution, by analyzing their variability over time and space. Furthermore, the study compared the health status associated with contamination in terms of heavy metals with that in two different areas of Italy, using hierarchical cluster analysis and principal component analysis, to evaluate the correlation existing among the three different areas of Italy. Following the findings, the authors suggest the use of honeybees as a bioindicator for heavy metal pollution in air quality studies.

Keywords: heavy metals; biomonitoring; pollution; bees; *Apis mellifera*; HCA; PCA

Citation: Di Fiore, C.; Nuzzo, A.; Torino, V.; De Cristofaro, A.; Notardonato, I.; Passarella, S.; Di Giorgi, S.; Avino, P. Honeybees as Bioindicators of Heavy Metal Pollution in Urban and Rural Areas in the South of Italy. *Atmosphere* **2022**, *13*, 624. https://doi.org/10.3390/atmos13040624

Academic Editors: Daniele Contini and Francesca Costabile

Received: 31 March 2022
Accepted: 12 April 2022
Published: 13 April 2022

1. Introduction

Heavy metals have attracted the interest of scientific communities for many years. They represent, in fact, a significant risk for the environment, considering the potential drastic effects deriving from their deposition and movement through soil, water, and air [1]. A study by Briffa et al. reported that the most common heavy metals in humans' everyday lives are vanadium, chromium, cobalt, nickel, copper, molybdenum, titanium, manganese, iron, and arsenic [2]. These inorganic substances are naturally present in the environment but, due to the astounding increase in the use of heavy metals, the presence of heavy metals in the terrestrial (and also aquatic) ecosystem has significantly increased. Industrial activities are responsible for the emission of heavy metals; products used in agricultural activities, such as herbicide, fertilizers, and insecticides, have led to an increase in environmental pollution. For this reason, the monitoring of the environment's health status is required [3]. From the available scientific literature, it is known that most traditional methodologies for environmental monitoring can cover only a small area of interest. Furthermore, the costs for maintaining and installing the necessary instruments onsite are high [4]. On the basis of this consideration, living organisms such as plants and insects have been taken into account [5]. The living organisms used for the biomonitoring can be classified depending on the type of interaction with the pollutant [6]. Among biomarkers, it is possible to distinguish between the bioindicators and the bioaccumulators. The first group consists of living organisms that show lesions and scarce diversity in a given area resulting from the impact of pollution. These aspects can be studied by means of specific growth models. The bioaccumulators (e.g., lichens) are a group of organisms that show significant changes in their morphology when exposed to contaminants. These organisms are able to survive the presence and the assimilation of contaminants. Due to their long life, they are capable of storing in their tissues an important quantity of contaminants, thus enabling quantification and qualification of the impact of anthropogenic activities on the environment [7,8].

Various studies have used living organisms, particularly honeybees, as indicators of the presence of several contaminants in the environment [9].

Environmental biomonitoring by means of the honeybee—in addition to other insects—dates back to Svoboda, 1961, and one of the first applications was for the monitoring of the presence of arsenic in certain areas of Czechoslovakia due to industrial pollution [10]. The first time that honeybees were used as bioindicators for heavy metal detection was in 1970 [11].

The use of honeybees as a bioindicator has increased during the last few years. For example, in their work, Negri et al. used *A. mellifera* for monitoring a polluted area in South-West Sardinia (Italy) that is also exposed to dust emissions from industrial plants.

By analyzing the bees by means of scanning electron microscopy coupled with X-ray spectroscopy (SEM-EDX), Negri and his co-authors demonstrated that inorganic particles were localized in specific locations of the honeybees' body, such as along the costal margin of the fore wings, the medial plane of the head, and the inner surface of the hind legs, due to the handling of the pollen [12]. Furthermore, the presence of an important number of inorganic substances and airborne dusts was found in epicuticular wax and the hairs. This phenomenon may have been due to air flow, which entrapped these particles, canalizing them in those locations of the honeybees' body. Furthermore, a work by Pellecchia et al. confirmed the importance of *A. mellifera* L. as a detector of air pollution, in particular for air particulates. Forager bees, during their wide-ranging foraging activity, collected samples of airborne particles emitted from a cement factory located in a rural landscape in Val d'Arda (Piacenza, Italy) [13]. Due to the wide flight range of honeybees (of up to 7 km^2), they can store and accumulate pollutants [14]. During foraging flights, honeybees are thus exposed to a wide range of pollutants, which are retained by their body hair [15]. Therefore, they may be able to highlight anomalies in the environmental distribution of heavy metals and their changes not only in time but, most significantly, in space [16]. In particular, in the case of heavy metals, direct measurements of bioaccumulation from *A. mellifera* L. are suitable since these chemical species are characterized by a slow and non-immediate toxicity mechanism [17].

However, papers that deal with the biomonitoring of heavy metals using *Apis mellifera* L. are still limited. As a result, the authors undertook the current research activity in order to expand the existing scientific knowledge about the use of living organisms as bioindicators.

Due to their wide-ranging foraging behavior, honeybees are able to detect and monitor environmental pollution. Compared to other studies, which dealt with a limited range of heavy metals and significantly different locations in terms of pollution, but did not repeat the sampling for a long period [16], this work not only tried to determine eight heavy metals, but also to analyze five locations with high, intermediate, and low levels of pollution for a prolonged period. Firstly, in this study, the authors specifically investigated the level of pollution in five areas characterized by a potential rate of increasing pollution (a green area, urban area, and rural area). This choice was based on a biological consideration of bees. From the scientific literature, it is known that the content of elements in bees is influenced by various aspects such as: beekeeping area (including types of soils and nectar plants) and its ecological status; breeding methods of bee colonies (incl. supplementary); age of worker bees; and physiological and health status of bee specimens and bee colonies. A work by ZhelyaZkova (2012) reported that the change in heavy metal content in the bee is influenced by the sampling region [18]. Therefore, considering the presence of a non-polluted area (or potentially such), the authors argue that it is possible to trace the anthropic impact on the release of metals into the environment. This latter aspect is particularly important for the authors. Briffa et al. reported that the main source of heavy metals is anthropogenic activities (i.e., foundries and other industries that are metal-based, automobiles, roadworks, and agriculture) [2]. For this reason, the possibility of distinguishing between natural and anthropic emissions of heavy metals into the environment may be useful for implementing measures for their control.

This study focused on the determination of heavy metal concentrations in urban and rural areas of Molise Region (South of Italy) by analyzing *Apis mellifera* L. samples. The sampling period was between March and September 2017 because of the major activity of honeybees, and it was carried out over a 7-month period in five areas of Molise Region. This work also aimed to compare the general environmental health status of Italy, in terms of heavy metal contamination, using living organisms.

2. Materials and Methods

2.1. Areas of Investigation

In this work, *Apis mellifera* L. samples were collected from five areas of Molise Region (Figure 1).

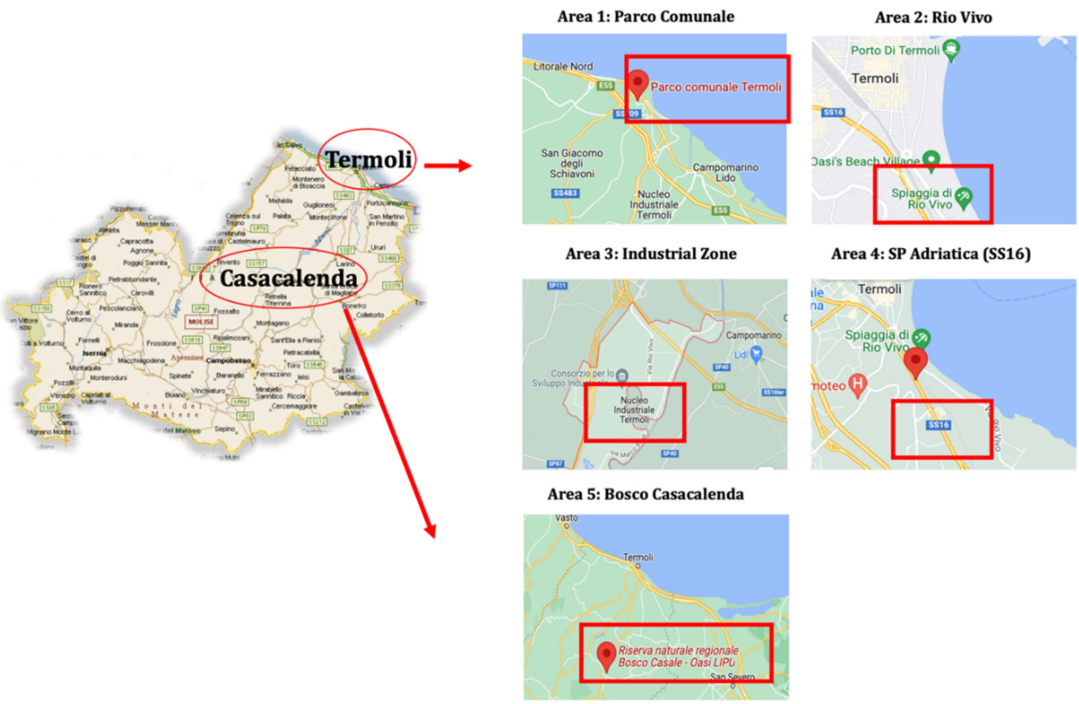

Figure 1. Maps of the areas of investigation, showing the zones in which honeybee sampling sites were placed (highlighted by the squares in red). Kindly supplied by Google Maps and modified.

The total analyzed area has a size of about 10 km^2, characterized by different levels of heavy metal pollution.

The first area (Area 1: Parco Comunale) is the Municipal Park of the city of Termoli (Molise, Italy), a large green area in the center of the city. The second investigated area (Area 2: Rio Vivo) is a seaside resort. The sampling was also carried out at an industrial area (Area 3: Industrial Zone) of Termoli and at State Road SS16 (Area 4: SP Adriatica 16), which is a fast road, characterized by vehicular traffic. As a non-polluted area, the authors considered a green zone of Molise (Area 5: Bosco di Casacalenda), which hosts the Lipu Oasis. Some of these areas are major tourist attractions during the summer period (such as Areas 1, 2 and 5), whereas Areas 4 and 5 are exposed to emissions from industries and vehicular traffic.

2.2. Sample Collection and Preparation

For determining the level of heavy metal pollution, hives, equipped with cages for dead bees, were used instead of traditional monitoring stations. The sampling activity was carried out from March to October 2017, every 15 days on average. The sampling was limited to the dead honeybees. After the sampling activities, honeybees were stored at −20 °C in a freezer; each sample was analyzed within 30 days of the sampling.

2.3. Heavy Metal Analysis

In this work, a deep investigation of heavy metals, such as beryllium (Be), cadmium (Cd), cobalt (Co), chromium (Cr), nickel (Ni), lead (Pb), copper (Cu), and vanadium (V), from samples of *Apis mellifera* L. was carried out.

A standard solution of heavy metals in a concentration of 20 µg mL^{-1} was purchased from Ultra Scientific (Bologna, Italy). Nitric acid with a purity ≥69% (Charlotte, NC, USA) and ultrapure water, obtained using a Milli-Q system (Millipore, Bedford, MA, USA), were used for the chemical analysis of heavy metals.

Heavy metal extraction from *Apis mellifera* L. samples was carried out using the procedure following EPA method 3050B [19]. Briefly, 4 g of each honeybee sample was weighted in a porcelain capsule and then placed in a muffle at 130 °C. After an hour, an increase of 50 °C was determined, up to 380 °C, in order to achieve the constant mass of the sample. The sample was then placed into a falcon (50 mL) for cooling, and recovered with 5 mL of nitric acid (5%). A ED36S Digiblock digester (LabTech, Boston, MA, USA) was used for the mineralization of the sample for 30 min at 120 °C. The cooled samples were then diluted with ultrapure water, up to a final volume of 25 mL, and analyzed with an ICP-OES (5110 Agilent Technologies, Santa Clara, CA, USA) according to the EPA method 6010C [20], from which all the analytical conditions are reported and discussed. Each sample was analyzed in triplicate.

3. Results and Discussions

3.1. Heavy Metal Levels in the Five Sampling Sites

According to our research, the heavy metals present in the highest concentrations were Cu, followed by Cr and Ni (Figure 2). From the analysis of the graph, it can be inferred that vanadium was also present in significant concentrations; however, it was always below the LOD, which is equal to 50 µg kg^{-1}.

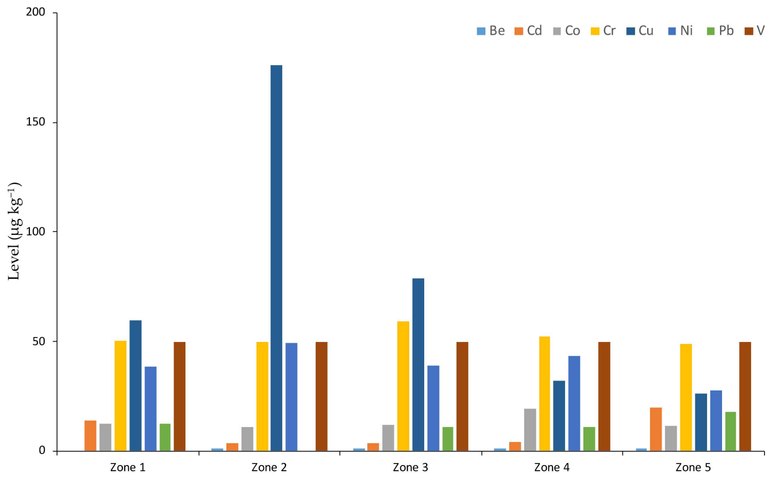

Figure 2. Average distribution of the investigated heavy metals in each zone. For sampling area description: see Section 2.1.

With the aim of identifying the potential source that determined a change in the concentration of each metal, and analyzing the suitability of honeybees as bioindicators, the authors analyzed the behavior of each chemical species in terms of time and space. Firstly, the authors analyzed the heavy metals' trends in Area 1, reported in Figure 3.

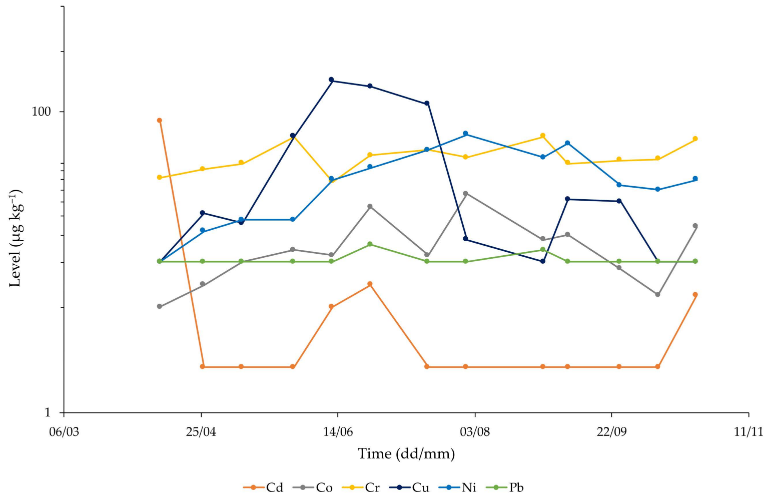

Figure 3. Trends in heavy metals in Area 1 over the sampling period. For sampling area description: see Section 2.1.

As shown in Figure 3, Cu increased during the months of May and August in zone 1. From the scientific literature, it is known that fertilizers, used for agricultural activities, contain heavy metals [21]. The Municipal Park (Zone 1) is surrounded by green areas and small villas. The increase in Cu in this area may also be attributed to the use of fertilizers for the cultivation of small home-run gardens. The application and use of fertilizers for crop control may result in an increase in Cu during the Spring–Summer period [22]. Cd presents an increasing concentration during the months of March and April, which was slightly higher than the LOD value (2 µg kg^{-1}) in subsequent periods.

A study by Yaaqub et al. reported that, on average, 52.5% of Cd, in terms of contamination, is provided by the atmosphere [23], whereas Ruschioni et al. explained that airborne Cd is transferred predominately by large-scale atmospheric transport [24]. For this reason, it is possible that the presence of Cd in the Municipal Park is due to a translocation of Cd particles from more polluted areas. Pb was slightly higher than the LOD value for all the sampling periods. Cr and Ni were revealed during all the sampling periods; the higher concentrations were achieved during the months of May, August, and September. Co was detected in all zones under investigation. The presence of Co in Area 1 may be related to energy production and vehicles that used new energy [25]. The presence of heavy metals in zone 1 may be caused by the fallout on the ground of airborne pollutants from the urban center, and emitted by various anthropogenic sources.

In Area 2, the heavy metal detected having the highest concentration was Cu. The increase was observed during the same period as that for Area 1, i.e., between May and August 2017 (Figure 4). From the scientific literature, it is known that high temperatures and dry climates can favor the increase of pollutants as they reduce the process of "leaching" of heavy metals from the flowers and, consequently, they can be more assimilated by bees. Our findings seem to confirm this, because a general increase in concentration can be observed during the hottest months of the year. In particular, in Area 2, the presence of Cu, Cr, and Ni is confirmed by biomonitoring. Pb was always below the LOD value, whereas Cd was detected in a low concentration (mean value 3.6 µg kg^{-1}). Co was detected in this area under study. Its presence may be due to the important vehicular traffic that characterizes this area.

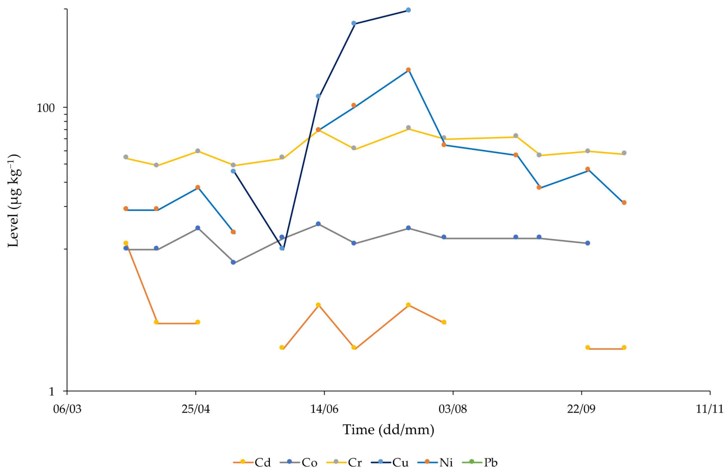

Figure 4. Trends of heavy metals in Area 2 over the sampling period. For sampling area description: see Section 2.1.

Figure 5 shows the trend in heavy metals throughout the sampling period in zone 3. Zone 3 is the industrial area of Termoli. An increase in the concentration of Cu was also observed in this area during the summer months. The authors hypothesized that this was due to the movement of pollutants from one area to another: in particular, from agricultural areas. From the scientific literature, it is known that this phenomenon can also occur for tens of kilometers and is favored by dry climates and high temperatures [26], which is in line with our findings. Cr increased in concentration during April 2017, whereas it remained constant throughout the remaining sampling period. Cd and Co were present during all of the sampling period in zone 3, with mean concentrations of 3.5 and 12 μg kg^{-1}, respectively. Our findings showed that Ni increased in concentration during the months of July and September 2017. According to the available scientific knowledge, Ni is released into the environment by several sources, such as steel and metal industries [27]. The industrial area of Termoli hosts metal industries, which can explain the presence of Ni in the environment. Regarding Pb, it was below the LOD during the entire period of sampling. However, a high concentration of Pb of 3305 μg kg^{-1} was detected in April. The authors decided to treat this value as an outlier, but further sampling in this area may be useful for more careful understanding of Pb emissions and contamination.

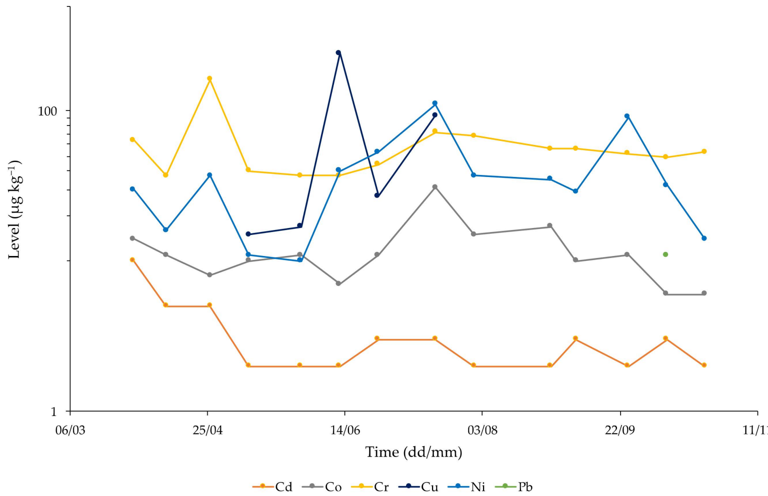

Figure 5. Trend in heavy metals in Area 3 over the sampling time. For sampling area description: see Section 2.1.

Figure 6 shows the trend in heavy metals throughout the sampling period in Area 4, which is a fast road characterized by intense vehicular traffic throughout the year. Our findings showed that Ni increased in concentration during the month of July 2017. According to the scientific literature, one of the main sources of Ni contamination is vehicular traffic, and particularly tire wear [27]. It may be supposed that, during the summertime, there was an increase in vehicular traffic, but this aspect needs to be confirmed. Cu also increased during the summer months in this area. This area is surrounded by cultivated land. It may be supposed that the increase in Cu is due to its use as a fertilizer in adjacent soils and to its displacement during the summer, which is in line with the scientific knowledge. As shown in Figure 6, Cr and Co were revealed throughout the sampling period, without showing significant increases in concentration. Cd was detected during all of the sampling period, whereas Pb was always below the LOD value (<10 µg kg^{-1}).

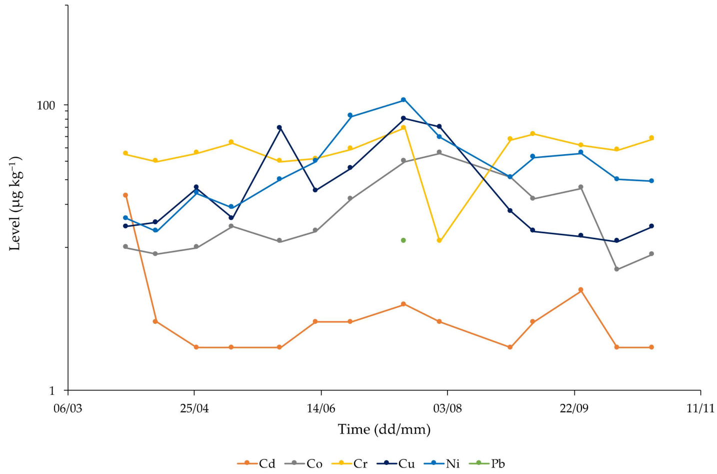

Figure 6. Trend in heavy metals in Area 4 over the sampling time. For sampling area description: see Section 2.1.

In contrast, Area 5 is a non-polluted area. Figure 7 shows the levels of heavy metals of the area under investigation. In this area, Cu was detected only during the months of May and June, in a mean concentration of 26.5 µg kg^{-1}. Pb was only detected during the first month of sampling, at a concentration of 18 µg kg^{-1}, whereas it was below the LOD during the remainder of the sampling period. Cd was present during September–October, at a mean concentration of 19.7 µg kg^{-1}. Ni, Cr, and Co were detected in this area for all of the period under investigation (mean concentrations: Ni 27.5 µg kg^{-1}; Cr 40.1 µg kg^{-1}; Co 13.5 µg kg^{-1}). The authors hypothesized that the displacement of heavy metals occurred. However, further investigations are necessary to better understand the sources of heavy metals in a non-polluted area.

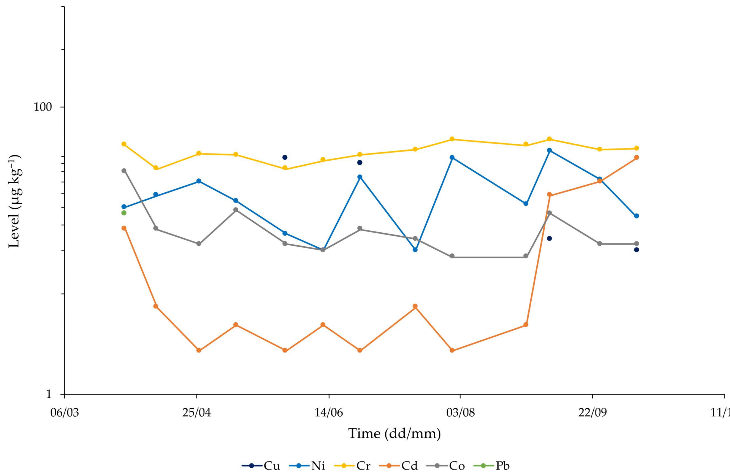

Figure 7. Trend in heavy metals in Area 5 over the sampling time. For sampling area description: see Section 2.1.

3.2. Comparison with Similar Studies

Several studies have focused on the biomonitoring of heavy metals in different areas of Italy. The authors compared their results, obtained in the South of Italy (Molise Region), with those present in the scientific literature relating to other areas of Italy, with the aim of evaluating the general health status of Italy in terms of heavy metal pollution. Before presenting the comparison, the authors would like to specify the importance of some particular elements, i.e., beryllium and vanadium, investigated in this study. Beryllium was considered because, from the scientific literature, it is known that this heavy metal can be a threatening heavy metal pollutant in the agro-ecosystem. Its presence in the environment is alarming for the productivity and sustainability of the ecosystem [28]. With regard to vanadium, in recent years, it has been considered to be a serious matter of discussion for the scientific community which deals with heavy metals. It can cause adverse effects in humans due to its mobility from soils to plants [29].

A study by Giglio et al. dealt with the heavy metal pollution in Trieste (Friuli Venezia Giulia region, Italy), using *Apis mellifera* L. as a bioindicator [28–30]. In this paper, two sampling areas were taken into account. Specifically, one site was located close to an industrial area and the second hive was placed 5.22 km away from an iron foundry, which can be considered to be a suburban area. Satta et al. focused on the biomonitoring of heavy metals using honeybees (and their products) in the Sardinia region (Italy) [29–31]. Three different sites were investigated. Specifically, two stations were placed close to small villages in Sardinia, and another was used as a control. Satta and co-authors carried out an investigation in a mining area, located in the southwest of Sardinia, characterized by a lead and zinc metallic vein.

For a deeper knowledge about correlations among the sites analyzed in our paper and the others presented in the scientific literature, a statistical analysis was carried out based on hierarchical cluster analysis and principal component analysis. The statistical analysis was conducted with the software Past, using the Pearson's correlation and Euclidean distance as measures.

First, the authors carried out a statistical analysis for evaluating the correlations among the five sites investigated in the present study and the sites analyzed by the other papers, as reported in Table 1.

Table 1. Correlations among the five sites investigated in the present study and among the areas in Sardinia and Friuli Venezia Giulia regions. For the sites: see the text.

Area vs. Other Site	Correlation
Area 5 vs. Urban	−0.0685
Area 5 vs. Suburban	−0.0768
Area 5 vs. Site A	−0.2344
Area 5 vs. Site B	−0.1240
Area 5 vs. Site C	0.5374
Area 5 vs. Area 1	0.8550
Area 5 vs. Area 2	0.1781
Area 5 vs. Area 3	0.6415
Area 5 vs. Area 4	0.8301

Table 1 shows that there was a strong correlation among the Municipal Park of Termoli (Area 1), Rio Vivo (Area 2), the Industrial Zone of Termoli (Area 3), SP Adriatica (Area 4), and Area 5 (Bosco di Casacalenda), which represented our non-polluted area. Furthermore, there was a good correlation among our blank and "Musei" (Site C), which was the area used as the control in Satta et al. This may indicate that, among the two areas, there was a similar source of contamination. Different sources of contamination were instead identified for the urban and suburban sites analyzed by Giglio et al. in the Friuli Venezia Giulia region [30], and for site A and site B investigated by Satta and co-authors in the Sardinia region [31]. This may indicate that the emission of contaminants into the environment of Italian's regions was due to different sources.

The authors used Pearson's correlation in order to analyze the correlation between the investigated heavy metals among the sites, as shown in Figure 8.

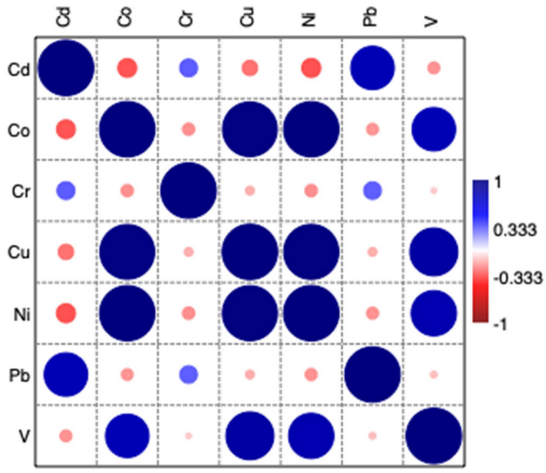

Figure 8. Pearson's correlation of investigated heavy metals among the sites of Molise (this study), Friuli Venezia Giulia [30], and Sardinia regions [31].

The heavy metals of the different sites are correlated with each other. This indicates that, although the sites analyzed are distributed in different areas of Italy, they are affected by a similar anthropogenic impact.

This is a multivariate problem; in fact, the characterization of the geographical areas by a wide chemical profile is a typical example of statistical multivariate analysis. A chemometric investigation was carried out to confirm the information reported above. To achieve this aim, hierarchical cluster analysis (HCA) and principal component analysis (PCA) were used; the characteristics of the five areas investigated were reported as geographical factors, whereas the variables were the eight heavy metals determined in the bee samples.

The authors carried out HCA to underline the evidence in terms of correlation among the investigated sites and the other sites presented in the scientific literature. As can be seen in Figure 9, the five sites investigated in the present study are clustered in one group and the two sites investigated by Giglio and co-authors are clustered in another group, whereas the three sites investigated by Satta are clustered into two different groups. In particular, site C, which was the blank used by Satta and co-authors, was similar to our sites, confirming the previously analyzed correlation. This may indicate that the sources of contamination among the three analyzed Italian regions are different. By means of the hierarchical cluster analysis, it is possible to hypothesize that the sources that determined the pollution phenomena in Molise Region are the same among the sites.

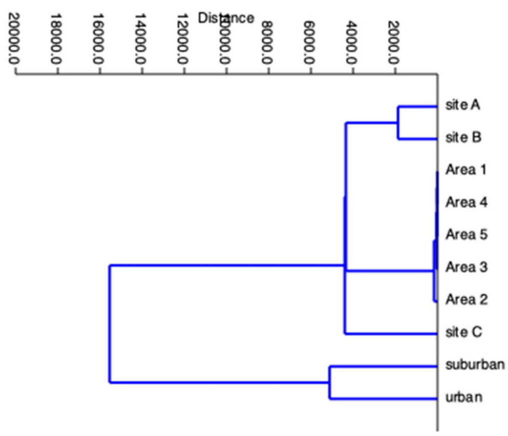

Figure 9. Dendrogram, obtained by means of hierarchical cluster analysis, showing the similarities among the sites investigated in this study.

On the basis of this consideration, the authors applied PCA to all data, as shown in Figure 10.

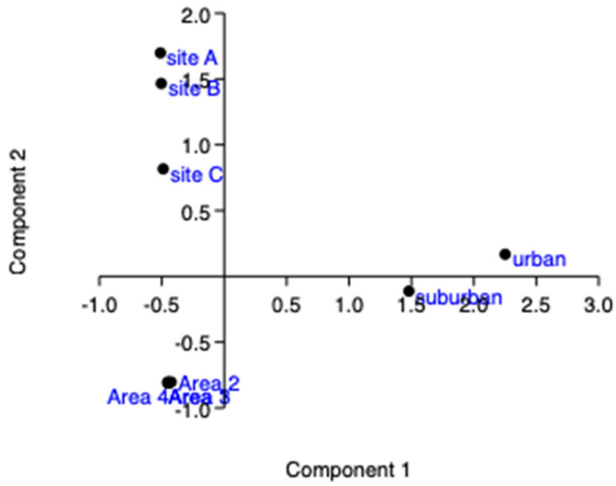

Figure 10. Principal component analysis applied to sites from Friuli Venezia Giulia, Sardinia, and Molise Regions.

First, three parameters are able to describe 99% of the overall data. On the first component, it is possible to find the urban and suburban sites analyzed by Giglio and co-authors, whereas Molise's sites and sites A, B, and C from the work by Satta are present on the second component. All these data confirm that the anthropogenic impact in the three Italian regions is similar, but the sources of contamination are different. The sites analyzed in our work, in fact, are mainly exposed to vehicular traffic, industries, and agricultural activities. Although these are all anthropic activities, they differ from those of the mining area analyzed by Satta and the iron industry close to the sites analyzed by Giglio. Our analysis confirmed the important and significant contribution of human activities in terms of the release of heavy metals into the environment, while also confirming the differences in terms of sources.

4. Conclusions

The present study aimed to highlight the suitability of the honeybee (*Apis mellifera* L.) as a bioindicator of heavy metals' environmental pollution. As shown by our findings, the honeybee is able to collect the pollutants present in the environment, and to provide information about the state of environmental pollution over time and space. The graphs reported showed the capability of the honeybee to record the variability, in terms of concentration, in heavy metals in the environment. The authors believe that this approach would be useful for a better understanding of the sources of contamination. Furthermore, a comparison with two other areas of Italy was undertaken, which was useful to (i) understand the general health status of Italy, in terms of heavy metal pollution, and (ii) confirm the suitability of honeybees as environmental pollution bioindicators.

Author Contributions: Conceptualization, A.D.C. and P.A.; methodology, P.A.; software, I.N., S.P. and S.D.G.; validation, C.D.F. and V.T.; formal analysis, A.N. and V.T.; investigation, I.N. and S.P.; resources, A.D.C.; data curation, C.D.F., A.N. and V.T.; writing—original draft preparation, C.D.F.; writing—review and editing, A.D.C. and P.A.; visualization, C.D.F.; supervision, A.D.C.; project administration, P.A.; funding acquisition, A.D.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by GAL Molise under the project "Bio monitoraggio del territorio con api sentinelle", grant number "PSR 2014–2020 Misura 19.2.16".

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors would like to thank ARPA Molise for their help in the collection data.

Conflicts of Interest: The authors declare no conflict of interest.

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