



# Bioindicators and biomonitoring: honeybees and hive products as pollution impact assessment tools for the Mediterranean area

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## Abstract

The global range of the environmental quality issues we all face necessitates integrated action from all of the countries that suffer from a particular environmental issue. We must be able to obtain data on the presence of pollutants and their effects on biota from any territory, regardless of its environmental, infrastructural, social, and economic conditions. Biomonitoring utilizes organisms and natural materials to obtain this information. In particular, the honeybee is a ubiquitous, easy-to-breed organism with great mobility. Its body, which is covered with hairs, picks up materials and particulates that it encounters in the environment. Therefore, bees are highly effective accumulators of materials from the soil, vegetation, air, and water. These characteristics mean that the honeybee is both a bioindicator and a passive bioaccumulator organism, making it an ideal agent for easily monitoring vast areas inexpensively, even in regions where infrastructure is scarce. In this short review, we summarize the main targets of the honeybee-based monitoring campaigns that have been carried out to date, highlighting the results obtained in assessments of organic and inorganic pollution performed by coupling more modern technologies with this long-standing practice. It is hoped that this review will make scientists more aware of the incredible potential of these delicate organisms to provide data that could prove useful in the management of environmental issues.

**Keywords** Bioindicators · Environmental monitoring · Honeybees · Pesticides · Heavy metals · Organic pollutants · Radionuclides

## Environmental biomonitoring

Widespread anthropization and various human activities are degrading the environment with increasing intensity. Our awareness of the incalculable damage, including serious diseases, caused by continually discharging chemicals into

the environment has grown considerably over the last few decades, especially in relation to persistent and accumulating pollutants. Nevertheless, the number of toxicants that have been released into the environment is now very large, and only a tiny fraction of those substances are regularly monitored. In recent decades, environmental directives have lowered the permitted levels of pollutants in water, the air, and foods, and have lengthened the list of chemicals that are banned and need to be assessed (US EPA 1970, 1978, 2016; EU 2000, 2008a, b, 2010, 2015, 2018; POPRC 2016). Due to the unquestionable importance of aquatic resources, the conservation and sustainable management of aquatic ecosystems is a priority in environmental programs worldwide. Similarly, air pollution is a serious global problem that threatens ecosystems as well as human health and life (US EPA 1974, 2000). Air contaminants reach the soil and water bodies where they—together with the xenobiotics employed in intensive agricultural activities—can enter the food chain, producing further damage (EU 2008b).

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One of the main challenges in contamination surveillance is to comprehensively characterize and evaluate the impacts of the presence of a complex mixture of pollutants on the environment. New monitoring and analytical methods that exploit the most advanced technologies are continuously being developed and validated as possible tools for performing this highly demanding analysis (Ma et al. 2020; Ye et al. 2020; Ramachandaran et al. 2019; Li et al. 2019; Rasheed et al. 2019; Shi et al. 2019; Hernandez et al. 2019; Horne et al. 2020). Nevertheless, the quali-quantitative determination of xenobiotics is only part of the information needed to implement an accurate risk assessment.

Environmental pollution can be evaluated directly by applying a specific sampling method, transporting the resulting samples to the laboratory, and then analyzing them with a precise analytical procedure. These active procedures are accurate and efficient but involve various drawbacks. The concentration of a pollutant varies spatiotemporally, especially in air. Any pollution monitoring program should therefore be statistical in nature, and should include a high density of sampling points to guarantee representative results. However, establishing an array of monitoring instruments in the field is sometimes difficult due, for instance, to high equipment costs, the need to supply power to the device, and the requirement for attended operation. All of these factors can strongly limit the sampling point density, meaning that the resulting data can have low statistical value.

The development of passive sampling techniques based on free analyte flow from the environment to a collecting medium has yielded low-tech, low-maintenance, and cost-effective methods for environmental monitoring that do not require a power source. Nevertheless, passive techniques based on substance capture by membranes or polymers cannot detect short-term variations in pollutant concentrations or environmental conditions, and they can present calibration problems (Lai et al. 2019).

Passive sampling methods can also utilize biological species (organisms and materials) that can act as reliable sensors of pollutants in their habitats; this approach to environmental contamination surveillance is called “biomonitoring” (Butterworth et al. 1995; Wolterbeek 2002). Effect-based methods such as bioassays and biomarkers are essential tools for implementing monitoring strategies. The utility of living organisms as indicators of environmental quality has long been widely recognized, since—in contrast to chemical analysis—they can be used to directly examine the effects of pollutants on the biosphere. Over the past few decades, various plants, animals, fungi, and bacteria have been employed as effective bioindicators and biomonitors in air, soil, and water pollution surveys (Butterworth et al. 1995; Wolterbeek 2002; Al-Alam et al. 2019; Devillers and Pham-Dellegue 2002; Asif et al. 2018; Panichev and McCrindle 2004;

Storelli and Marcotrigiano 2005; Mahapatra et al. 2019; Van Meter et al. 2019).

Biomonitoring can also be classified into passive and active techniques. Passive biomonitoring is the observation and analysis of organisms that are indigenous to the ecosystem, whereas active biomonitoring involves the insertion (under controlled conditions) of organisms into the site to be monitored. Biomonitoring allows detailed and reliable coverage of a wide territory at relatively low cost—definitely lower than the cost of maintaining electronic monitoring stations. Moreover, the data obtained by biomonitoring are not comparable to that provided by a single sampling instrument, since a biomonitoring dataset provides information on the responses of a wide range of living organisms in the surveyed area to contaminants. Also, any instrumental or analytical chemistry measurement technique can be used to extract biomonitoring data. These advantages of biomonitoring make this approach to environmental contamination surveillance very useful.

Biomonitoring can employ sensitive or accumulative organisms: bioindicators or bioaccumulators, respectively (Wolterbeek 2002). Bioindicators display very high sensitivities to pollutants. They respond to the quality of the environment under examination by undergoing morphological and/or physiological changes, such as photosynthetic and/or respiratory activity modifications (Butterworth et al. 1995; Al-Alam et al. 2019; Batzias and Siontorou 2007). According to the US Environmental Protection Agency report on the environment (US EPA 2007), the key criteria for identifying a useful bioindicator are as follows:

- The indicator is useful, objective, reliable, and reproducible
- The underlying data are characterized by reliable collection methodologies, data management systems, and quality assurance procedures
- The data obtained can be used to derive changes or trends, are comparable across time and space, and are representative of the target population.

An organism must also have the following characteristics to be able to act as a bioindicator:

- Easily identified and easily sampled
- Is widespread in the studied area and has low mobility
- Has a long life cycle and shows high genetic uniformity across the studied area
- Is present in the studied area all year long.

Bioindicators permit the establishment of a network of monitoring sites with satisfactory coverage of the territory of interest. They do not require sophisticated and expensive equipment, an electricity supply, or specialized personnel.

They allow a region to be probed for structural damage (either reversible or permanent) and to be monitored for any decrease in biotic diversity, i.e., a decrease in the number of species in the community along with a parallel increase in the number of individuals belonging to the more resistant species. The information they have supplied has raised our awareness of the effects of environmental pollution. Thus, the availability of a large number of reliable bioindicator species is an important step towards the wider application of this methodology.

An organism is defined as a bioaccumulator when it stores contaminants in its tissues. Bioaccumulation is an equilibrium process in which the bioaccumulator takes in and discharges substances from and into the surrounding environment. The mode of deposition of a pollutant in the environment can be inferred from its concentration in the bioaccumulator organism.

The disadvantages of some bioaccumulation techniques include the need for complicated analytical equipment and a detailed understanding of the taxonomy of one or more groups of organisms. Moreover, cumulative effects cannot be used to inform short-term decision-making and compensatory actions (Batzias and Siontorou 2007). When the pollutant is also a substance or an element that is present in the bioaccumulator naturally, it is necessary to assess the background value of that chemical by analyzing a blank (i.e., the same bioaccumulator but from an unpolluted area).

Some organisms that are typical of the environment under study can act as both a biosensor and a bioaccumulator; this is the case for honeybees.

## Honeybees and hive products as bioindicators and bioaccumulators of environmental pollution

Worldwide, the genus *Apis* (honeybees) includes several species (see Table 1). The most well-known species of honeybee are those raised to produce honey and enhance the pollination. All honeybees organize themselves into societies that include both sexes. Females show morphological and physiological differences according to their specific roles.

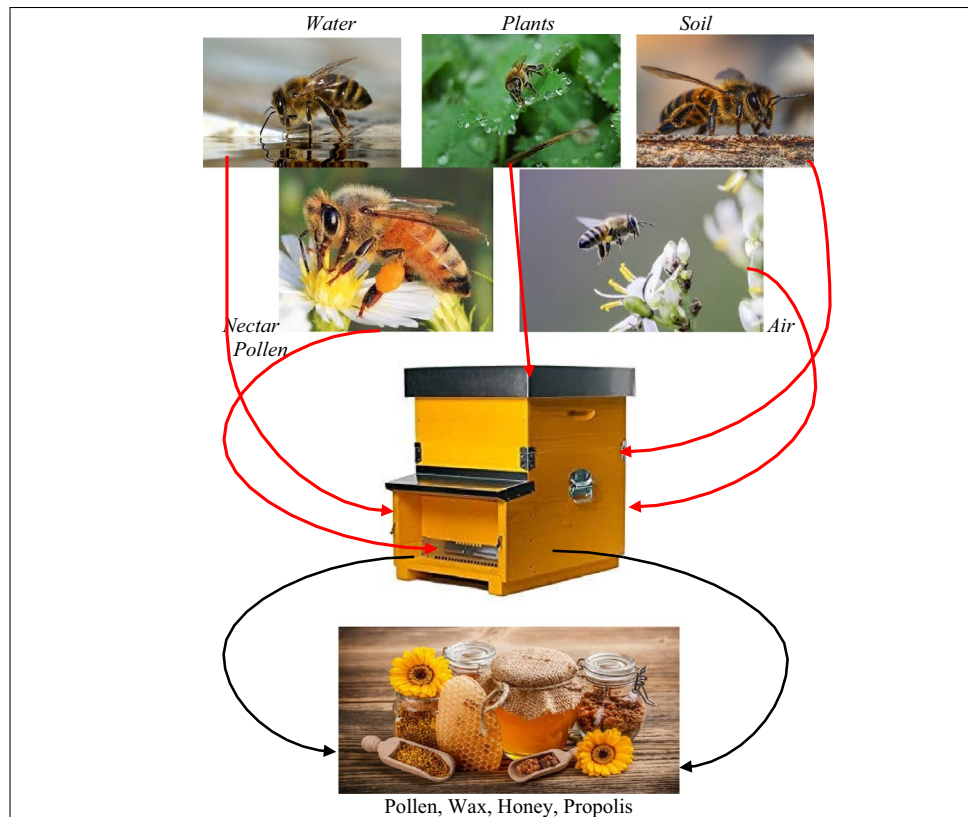
The honeybee (*Apis mellifera* L.) has proven to be a good biological indicator that is easy to use for environmental biomonitoring at any scale (Porrini et al. 2002). Honeybee-based biomonitoring is currently performed in many Mediterranean countries, such as Spain (Gutiérrez et al. 2015), Italy (Perugini et al. 2018), Croatia (Barišić et al. 2002), France (Badiou-Beneteau et al. 2013; Cotton et al. 2014), Greece (Kasiotis et al. 2014), Turkey (Yarsan et al. 2007), Iran (Sadegh et al. 2012), and Egypt (Malhat et al. 2015), as the favorable Mediterranean climate allows honeybee activity to be exploited for most of the year.

This organism is easy to breed, almost ubiquitous, and has modest food requirements. Its very high rate of reproduction and relatively short average lifespan causes honeybee colonies to undergo rapid, continuous regeneration, which plays a fundamental role in colony survival. These organisms do not accumulate and retain pollutants in their tissues for long periods; they transfer the compounds they collect to their products, such as honey, which is also used as a biomonitoring tool. The high mobility and wide flying range of honeybees allow a vast area to be monitored using these insects. The honeybee body is covered with hairs, which make it particularly suitable for capturing the particulate materials they encounter during their interactions with the environment.

**Table 1** Important species of *Apis* Linnaeus, 1758

<i>Apis</i> Linnaeus, 1758 species	Geographical distribution	Nest structure
<i>Apis mellifera</i> Linnaeus, 1758	Originally Europe, but now all over the world	Honeycombs within buried cavities
<i>Apis mellifera ligustica</i> Spinola, 1806	Italy	
<i>Apis andreniformis</i> Smith, 1858	Thailand, Vietnam, and Malaysia	Single honeycomb
<i>Apis laboriosa</i> F.Smith, 1871	Nepal, Laos, and China	Honeycombs on open-air rocks
<i>Apis nigrocincta</i> Smith, 1861	Indonesia	Honeycombs within buried cavities
<i>Apis cerana</i> Fabricius, 1793	Russia, New Guinea	Honeycombs within buried cavities
<i>Apis dorsata</i> Fabricius, 1793	India and Indonesia	Honeycombs on protruding rocks
<i>Apis florea</i> Fabricius, 1787	Southeastern Asia	Single honeycomb
<i>Apis cerana nuluensis</i> Tingek, Koeniger & Koeniger, 1996	Borneo	Honeycombs within buried cavities
<i>Apis vechti</i> (Maa, 1953)	Sumatra and Java	Honeycombs within buried cavities

**Fig. 1** Honeybees can capture pollutants that are in the air, deposited on plant surfaces and flowers, in the soil, and dissolved in water



Honeybees are a highly efficient means of performing ground surveys (they make numerous inspections per day), and the majority of their flights are within 2 km of their colony. They visit almost all environmental sectors (soil, vegetation, water, and air) within a circular area with a radius of approximately 12 km<sup>2</sup> around the hive, providing numerous randomly collected samples. They gather nectar, pollen, honeydew, propolis, and water, all of which are brought into the hive and stored there according to verifiable criteria. Once in the hive, these products are easy to sample and subsequently analyze for contaminants (i.e., the bees act as bioaccumulators) (see Fig. 1).

On the other hand, honeybees are highly sensitive to most agrochemicals. Bee mortality can greatly increase in the presence of certain highly toxic compounds, which immediately highlights the forbidden spread of such chemicals throughout the environment (e.g., during flowering, in the presence of wild blooms, during windy days, etc.). Physiological modifications can appear in bees after they have ingested less toxic pollutants, which can be identified by chemical and biochemical analyses (i.e., the bees act as biosensors). Hence, bees can be used to detect chemical impairment of their habitat by studying various endpoints: mortality (in the case of pesticides to which they are very

sensitive); physiological, biochemical, or behavioral changes; and the response of the population distribution to sublethal pollutant toxicity (Dolores Hernando et al. 2018). The levels of residues on or within their bodies and the contamination of beehive products reflect the pollutant content of their environment (Porrini et al. 2002; Badiou-Beneteau et al. 2013; Codling et al. 2016; Cousin et al. 2019; Drummond et al. 2017; El-Saad et al. 2017; Hernandez et al. 2019).

Note that, henceforth, when we mention “honeybees,” we are referring specifically to worker bees, as they are the only component of the beehive that carries out foraging activities and thus undergoes multiple interactions with both the environment and the hive.

The whole bees plus hive products system represents a very interesting and economical way to obtain samples that are useful for assessing contamination levels and their effects at the same time. Beehives are inexpensive and do not require a power supply, so they can be placed at any location. Beekeeping has been practiced worldwide since ancient times, and specialized personnel are not required to carry out biomonitoring using bees, except for those who are needed to perform and interpret analytical procedures (Van der Steen 2016). Given that the production of 1 kg of honey requires more than 100,000 foraging flights,

it is clear that both honeybees and honey are appropriate random samples, so results obtained from honeybee-based monitoring will be highly representative of the average levels of accessible pollutants in the monitored area. Finally, honeybees are indispensable pollinators that are of great importance to ecosystem integrity. As they are bioindicators, honeybees can be used to assess and monitor changes in agricultural ecosystem quality and can supply ecological data (Quigley et al. 2019).

### The monitoring protocol

Effective environmental biomonitoring requires programs that provide specific links between changes in environmental conditions and ecosystem health. Biomonitoring can be carried out using honeybees at different levels of complexity and sensitivity. The financial cost and the skills needed to perform such biomonitoring increase with the frequency of sampling, the diversity of the samples collected, and the amount of equipment and number of analytical techniques employed, but increasing any of these factors will also result in more data of greater quality. On the other hand, increasing these factors will reduce the field of applicability for biomonitoring. Thus, the appropriate level for each of the factors depends on the context and the aims of the biomonitoring performed.

A honeybees–hives system can be employed to passively sample and concentrate pollutants in order to obtain data on environmental quality in three main steps: field sampling by the bees; subsampling of dead or alive bees and hive products; and analytical determination of the contaminants followed by data processing (Van der Steen 2016).

The simplest experimental design involves placing a certain number of hives inside the region of interest, while another group—used as a control—is placed in a completely or relatively unpolluted area. Nevertheless, monitoring environmental pollution using honeybees requires the application of statistical tools to determine the optimal experimental design, starting with the sampling networks and the sampling procedures (Pirk et al. 2013; Dietemann et al. 2016). The number of stations and the number of samples collected per station must be planned in advance based on the characteristics of the sampled area, the presence and distribution of pollution sources, and atmospheric conditions. Moreover, the size and the type of the samples (dead or alive bees, hive products) to be collected and analyzed are selected beforehand based on statistical considerations: a sufficient number of samples must be taken to achieve the required statistical precision. The sample treatments and analytical techniques applied influence the number of samples that can be analyzed. Statistical tools must be used to process the large amount of data obtained, including sample measurements, atmospheric, agricultural, botanical, and geochemical

information, and other useful data. Beyond the application of statistical tools to determine the significance of differences between the control area and the area suspected to be contaminated, ANOVA or paired *t* tests (for example) can be applied to parametric data, or a MANOVA test to multi-analyte data (Warne 2014). In fact, most of the analytical techniques used in honeybee-based biomonitoring are multielemental, so multivariate chemometric procedures such as principal component analysis or hierarchical cluster analysis are required to discern the relationships between various environmental variables and the contents of the samples (Yücel and Sultanoğlu 2012). Statistical methods can also be used to determine spatiotemporal variations in pollutant levels (Zaric et al. 2018).

Either dead or alive honeybees can be employed as the sampling population. In the former case, they are collected via cages, traps, underbaskets, etc. (Porrini et al. 2002), and the vegetation and areas visited by the bees are identified through pollen identification analysis. In nonsacrificial sampling of alive bees, the residues attached to their bodies are removed and collected on plastic surfaces on which the bees are forced to walk (Van der Steen 2016). Hive product sampling is always much easier. The best substrate to analyze depends on the kinds of analytes that are being investigated. Finally, the most appropriate sample treatment and analytical method will be employed to determine the chemicals encountered by the bees in their environment.

An alternative way of using honeybees to evaluate environmental quality is to look for changes in their behavior. It is possible to record the sound patterns at the hive, to monitor in-and-out movements of foraging bees, or to determine the number of bees using an infrared-based imaging system (Pérez et al. 2016; Chen et al. 2012; Shaw et al. 2011; Cousin et al. 2019). Laser-based optical systems that record the wing-beat frequency can be used to detect honeybee movements near the beehive or near a specific target, as well as those flying in the environment (Hoffman et al. 2007; Brydegaard et al. 2016). As an example, Table 2 reports the monitoring protocols we have applied in most of our studies.

### Current applications of biomonitoring using honeybees

Historically, honeybees and hive products were initially used to monitor contamination from heavy metals and radionuclides (Svoboda 1961, 1962), which show high similarity in terms of their chemical properties and dynamics in the biosphere. These contaminants are still monitored by analyzing bees and their products (Badiou-Beneteau et al. 2013; Nikolić et al. 2016; Gutiérrez et al. 2015; Skorbilowicz et al. 2018). Later, honeybees were most commonly used to



**Table 2** Monitoring protocols utilized to monitor different contaminants in our studies

Pollutant	No. hives/ station	Analytical matrix	Analytical methods employed	Supplementary data
Pesticides	2	Dead bees collected weekly from underbasket	SPME, GC, HPLC	Mortality threshold: 250 bees/week/ station Crop-growing maps Pollen identification
Heavy metals	2	Foraging bees captured every 15 days, and fresh honey collected monthly	Mineralization, atomic ionization Spectroscopy	Crop-growing maps Pollen identification
Radionuclides	2	Dead bees collected weekly from underbasket	Germanium crystal gamma spectrometry	Crop-growing maps Pollen identification

monitor the levels of pesticides in fields and on vegetables visited by honeybees (Quigley et al. 2019; Codling et al. 2016; Kasiotis et al. 2014; Barganska et al. 2018; de Oliveira et al. 2016). Several persistent organic pollutants (including explosive residues) originating from industrial and urban activities have also been isolated from bee samples and evaluated (Garcia-Valcarcel et al. 2016; Drummond et al. 2017; Simic et al. 2019; Gómez-Ramos et al. 2019). More recently, nanomaterials and nanoparticles have been added to the list of contaminants that honeybees can be used to monitor in the environment (Milivojevic et al. 2015; Kojic et al. 2020; Hooven et al. 2019; Glavan et al. 2017).

Since the beginning of the 1980s, our research group has been studying the best protocols for exploiting honeybees as bioindicators of environmental pollution, including pesticides, heavy metals, radionuclides, and phytopathogenic bacteria. The information supplied by palynological analysis and crop-growing maps was processed together with the chemical data in order to develop a predictive model suitable for environmental impact assessment (Porrini et al. 2002; Gentilomi et al. 2003).

### Heavy metals and elements

Unfortunately, there is no area of the Earth that is completely free from contamination with heavy metals (HMs) of anthropogenic origin (Badiou-Beneteau et al. 2013). HMs are continually released from various natural and anthropic sources and are not degraded, so they become trapped in physical and biological cycles.

Since these pollutants have become ubiquitous, it is impossible to find uncontaminated control areas. Atmospheric metal deposition can occur over large areas far from the industrial and urban sources of those metals (Van der Steen et al. 2015; Van der Steen 2016). Monitoring studies can be employed to pinpoint new contamination sources or the origin and spread of a specific element.

Moreover, particular cunning must be used to avoid contamination from the materials employed to prepare the hive,

or from the smoke used to calm bees, and finally from the containers used to collect, manipulate, or store the samples.

Regarding the choice of the most suitable matrix to analyze, most studies have studied dead bees and/or honey. In general, honeybees represent a better matrix for evaluating metal pollution than honey. Heavy metals in the atmosphere can be deposited on the hairy bodies of the bees and then brought into the hive with pollen, or they may be ingested with the nectar of flowers, water, or honeydew. A number of variables must be considered: the weather conditions, since rain and wind can clean the atmosphere; the nectar flow, which depends on the season; and the botanical origin of the honey, as aromatic plants concentrate pollutants and honey from deciduous trees has lower metal contents than honey from evergreen ones (Devillers et al. 2002; Yarsan et al. 2007). Thus, information obtained by palynological analysis on the botanical origin of honey can be fundamentally important. The soil composition can also affect the accumulation of metals in plants (Bogdanov et al. 2007).

By comparing atmospheric data with the results of bee analyses, it is possible to ascertain strong relationships between the abundances of metals in the environment and their levels on honeybees (Costa et al. 2019). Honeybees can provide additional information on heavy metal pollution, such as whether it is persistent or transitory. Various information is obtained by evaluating the data from samples obtained by washing the bees in acid (i.e., contaminants on the bees), data from samples containing only the metals ingested by the bees (obtained through wet acid digestion of the bees after washing their external surfaces), and data from samples containing the full body residues (obtained through simple mineralization of bees). As an example, during studies of the metal distributions in urban, industrial, and rural areas, we found that lead levels were higher inside the bees than on them ( $p < 0.0001$ ) in urban and industrial areas, whereas the opposite was true in rural areas ( $p < 0.0005$ ). For nickel, a significant difference between the levels found in and on the bees was only recorded in rural areas ( $p < 0.05$ ), where levels were higher on the bees. Finally, significantly

more chromium was found on than in the bees in all three environments (urban,  $p < 0.05$ ; industrial,  $p < 0.005$ ; rural,  $p < 0.005$ ). The higher levels of all three metals on rather than in the bees in rural areas may suggest that these metals are scattered in the atmosphere and do not penetrate into or deposit on the environmental components visited by bees, so they do not tend to ingest them (Porrini et al. 2002).

Finally, it is important to highlight the importance of using live bees to monitor metal contamination. Biomonitoring studies have shown that live bees are better for detecting high levels of heavy metals in the environment than dead ones (Ruschioni et al. 2013). Bees provide us with detailed information on recent changes in metal abundances, since the metals detected on them were collected during the previous 5 or 6 days (Porrini et al. 2002).

Honey and bee analyses can complement each other. A wide range of published papers have highlighted that honey is a suitable matrix for heavy metal detection (Herrero Latorre et al. 2018), but several studies have demonstrated that this matrix does not accurately reflect the environmental contamination level (Fakhimzadeh and Lodenius 2000; Conti and Botre 2001; Tuzen et al. 2007; Sadegh et al. 2012; Silici et al. 2013). The mineral content of honey has been found to be highly correlated with its geographical and botanical origins, its geochemical composition, and with local pollution levels. Thus, the composition of the honey can be considered to be indicative of a wide range of environmental factors (Sajtos et al. 2019; Fermo et al. 2013).

## Radionuclides

Small amounts of radionuclides can be dispersed into the environment by the chimneys of nuclear power plants, by health establishments, in army simulations of a nuclear accident, or by inappropriate disposal of radioactive sources in foundries (Porrini et al. 2002). However, the largest contributions to radionuclide contamination come from nuclear experiments performed in the atmosphere (Svoboda 1962) and leakages from or accidents at nuclear power plants. Radioactive particles that are dispersed into the air will gradually be deposited on soil and vegetables. Some of these deposited radionuclides are subsequently picked up by visiting insects, or they can be transferred into agricultural products or grazing animals, ultimately to be ingested by humans. The high suitability of honeybees and hive products for collecting radioisotopes from the environment was unequivocally demonstrated by several studies performed immediately after the Chernobyl accident. Those studies used bees and hive products to understand the distribution of the radioactive fallout and the transfer dynamics. Our research group analyzed numerous samples of honey, bees, wax, and pollen. The results demonstrated that pollen was the best indicator of atmospheric contamination levels, and

the bees themselves proved to be useful: measurements of the radionuclides in this matrix showed a strong correlation with ground deposition values ( $R = 0.95$ ). On the other hand, the radioactivity of honey was found to be greatly influenced by its pollen content (Tonelli et al. 1990). Soil composition and botanical origin also influence the radionuclide concentration in honey. Nevertheless, most of the studies carried out in the last 25 years have chosen honey as the matrix to analyze, probably because honey samples are the easiest to collect and the analytical technique is extremely sensitive and selective (Assman Assman-Werthmüller et al. 1991; Barišić et al. 2002; Panatto et al. 2007; Borowska et al. 2013; Meli et al. 2016). In fact, it is very easy to check for the presence of radionuclides in any kind of matrix without the need for any sample pretreatment. The bee matrix can be used to rapidly check for the presence of radioactivity in the atmosphere, even at levels that are far below every alarm threshold, with higher efficiency than traditional monitoring techniques (Porrini et al. 2002).

## Pesticides

These compounds are used extensively in agriculture. Newly developed families of pesticides are continuously spread on cultivated fields to control attacks from parasites that grow increasingly pesticide resistant. The side effects of these pesticides on untargeted organisms such as pollinators are nonnegligible.

Honeybees are extremely sensitive to pesticides. Bees capture insecticidal compounds mainly through the ingestion of water/liquids from a variety of sources, including ditches, puddles, canals, brooks, fountains, and dew. The hairs on their bodies intercept particles suspended in the atmosphere.

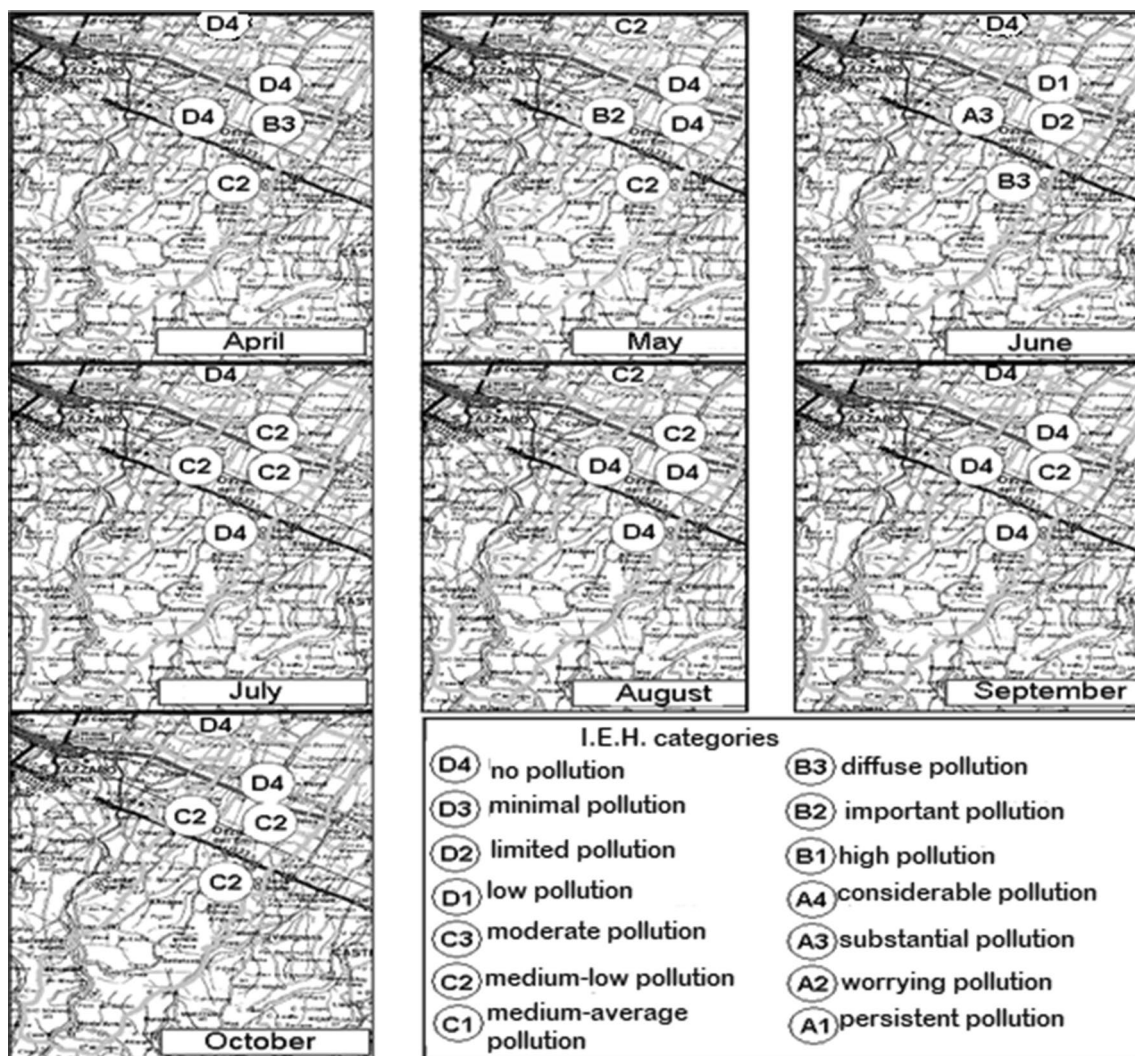
When captured, some compounds can be so toxic that the bees do not have enough strength to return to their hives; they die in the field or during their return flight. Bees that are only marginally damaged by a captured compound will eventually die in their hives. In this case, the honeybees act as direct indicators. The number of dead bees in front of the hive will vary according to the toxicity of the active principle (Atkins et al. 1981), the blooming of cultivated or spontaneous plants, the presence of honeybees during the chemical treatment, the means used to distribute the pesticide, the presence of wind, and other factors.

For compounds that are not particularly dangerous, the insect can act as an indirect indicator (i.e., it is not sensitive to the compound but it has been exposed to it), and can therefore provide us with information in the form of residues that we can analyze.

The identification of pollen in hive products or on the bees themselves, together with detailed knowledge of the vegetation present within the flying range of the bees, is







**Fig. 2** Examples of monthly maps of pesticide contamination in a territory around Bologna (Italy) that includes five monitoring stations. Each station was assigned monthly to an index of environmental hazard (IEH) category. The IEH is an indicator of environmental risk, since it is a combination of the honeybee mortality class (monthly

mean of dead bees/week) and the index of pesticide toxicity (IPT), which is calculated taking into account the toxicity and the persistence of the pesticide (or a mean IPT value when multiple pesticides are present) (Porrini et al. 2002)

96-well microplates via bonding with a specific DNA oligo probe. Detection was achieved using an immunoenzymatic enhanced chemiluminescent assay performed directly on the microplates (ECL-PCR-ELISA) (Merighi et al. 2000). The aim was to obtain an early warning system that can reveal the presence of this pathogen at trace levels in rosaceous pollen samples collected in seemingly uncontaminated areas, thus allowing sufficient time for intervention before the pathogen causes visible damage.

The samples analyzed were pollen and forager honeybees leaving the hive, with the pollen preferred due to ease of sampling. Palynologic analysis revealed that rosaceous plants were among those visited by the honeybees.

The monitoring stations are ideally distributed in circles centered on the infected areas. Alternatively, they can be aligned perpendicular to the disease expansion front. For example, we employed the latter approach, starting from the edge of the infected zone and separating the stations by 4 km up to 26 km from the edge. All of the areas monitored were considered to be unaffected based on visual inspections of plants, but our analysis of samples from several of the monitoring stations indicated the presence of *E. amilovora* in at least one sample/station. Due to the distance of the pathogen-positive monitoring station from the infected area, it took 4 months for visible symptoms of fire blight to appear on host plants close to that station, and more than a year for

host plants near to a station about 22 km from the original disease front to show signs of the disease.

### Explosives, nanoparticles, and other organic pollutants

Trace sensing of explosive vapor is a method that is used to detect mines and improvised explosive devices (IEDs). However, trace sampling in the field can be extremely difficult due to various factors, including the very low vapor pressures of the explosives of interest. It may be too risky to allow humans or dogs access to areas suspected of harboring mines. Honeybees can safely survey areas contaminated with landmines by collecting explosive materials on their body hairs, which electrostatically attract tiny particles of explosives suspended in the air. After the bees have returned to their hive, the particles and explosive molecules present on their bodies can be collected and concentrated.

The more or less successful detection of trace levels of explosives has also been achieved using another strategy: sniffer bee technology (Rodacy et al. 2002; Hadagali and Suan 2017), which involves training thousands of bees to seek the specific odors of 2,4-dinitrotoluene and TNT (Bromenshenk et al. 2003a, b; Anderson et al. 1999; Macias 2009). Honeybees have the ability to distinguish between than 170 odors; this ability is crucial to their ability to seek out nectar, pollen, water, tree resin, and pheromones (Halter 2011). In this case, honeybees are not employed for bio-monitoring but as highly sensitive, accurate, and cost-effective biosensors. This sniffer bee approach has been applied in various fields, such as theaters of war, during customs inspections to detect narcotics (Keith and Rooth 2012) and cigarettes (Wells and Bradley 2012), to support forensic investigations (Frederickx et al. 2011), and to discern food adulteration (Bonod et al. 2003).

As mentioned before, by determining the residues recovered from bees and hive product samples, it is possible to characterize the complete organic pollution patterns of the samples in just one analysis. Phthalates and PAHs (Gómez-Ramos et al. 2019), polychlorinated biphenyls (Bargańska and Namieśnik 2010), azoles and organophosphorus esters (García-Valcarcel et al. 2016), and a wide spectrum of other persistent organic pollutants (Wei et al. 2016) have been determined simultaneously in bees and hive products extracts. A comprehensive list of the pollutants present in the environment is necessary to correctly ascribe the assessed biotoxicity to its causative compounds.

Finally, the enormous proliferation of nanomaterials and their applications has led to these particles being placed on the list of environmental pollutants. Their spread across urban and rural areas has already put them into contact with honeybees. The toxic, or sometimes even beneficial, characteristics of nanomaterials such as ZnO, fullereneol,

nanotechnology-based pesticides, and silver nanoparticles coupled to plant extracts have been investigated (Milivojevic et al. 2015; Kojic et al. 2020; Hooven et al. 2019). The present studies were based on the particular adaptation of the pollinator to collect pollen, and then on the assumption that any other types of environmental particles can also be actively gathered by bees.

### Sample treatment and analysis

Honeybees, frozen or lyophilized after collection, are the most complex matrix to treat and to analyze. Pollen, propolis, and wax are simply stored in containers at room temperature under fresh and dry conditions. Honey and honeydew are stored in glass containers at 4 °C in the dark. Sample treatments and analytical techniques vary according to the analyte of interest.

### Elements and heavy metals

Metal determination is achieved using various atomic spectrometry techniques. Biological samples such as bees or honey must be mineralized before analysis. When studying bees, it is possible to analyze the metals deposited on the body separately from those ingested (together with nectar, water, and other liquids) by the bee. The metals on the bees can be collected by washing them with a 5% solution of acid (HNO<sub>3</sub>), whereas the ingested metals can be extracted by exposing the previously washed bees to wet acid digestion using various acids (HCl, HNO<sub>3</sub>, and H<sub>2</sub>O<sub>2</sub>) with or without microwave irradiation (Porrini et al. 2002; Lambert et al. 2012; Van der Steen et al. 2012; Silici et al. 2013; Gutiérrez et al. 2015). Honey can be mineralized or simply diluted, for example 1:20, in a 0.2% Triton X-100 water solution (Porrini et al. 2002).

A number of analytical techniques can be used to determine the levels of various metals, but the most popular (and suitable) are those based on atomic spectrometry (Pohl et al. 2012; Herrero Latorre et al. 2018). One of the most powerful of these is atomic absorption spectrometry, operated in either flame or electrochemical atomic absorption (EAA) mode. Electrothermal atomizers are advantageous in that they facilitate low limits of detection and require only very small sample volumes, which is useful when analyzing biological samples. When the metal concentrations are relatively high, flame atomic absorption can be sufficiently sensitive. Other techniques such as ICP-OES (inductively coupled plasma spectrometry–optical emission spectrometry) and ICP-MS (inductively coupled plasma spectrometry–mass spectrometry) offer the possibility of performing multielemental analysis in a single step. ICP-MS is the technique of choice when a high sensitivity is needed (Herrero

Latorre et al. 2018). A microwave plasma atomic emission spectroscopy (MP-AES) technique for the elemental analysis of honey samples has also been validated (Sajtos et al. 2019).

## Radionuclides

The qualitative and quantitative determination of radionuclides is always performed without any sample pretreatment due to the nature and high selectivity of the analytical technique applied. Each gamma-emitting radionuclide has a characteristic spectral intensity and energy, which makes gamma-ray spectrometry (GRS) using a low background hyper-pure germanium semiconductor detector and a multi-channel analyzer an extremely selective and sensitive technique. Even very low levels of radioactivity can be detected, including the background emission from natural radionuclides, and comparisons of data from different laboratories are possible (Gilmore 2008; Meli et al. 2016).

## Pesticides and organic pollutants

Analyzing these kinds of contaminants requires their careful and complete separation from highly complex organic matrices (i.e., bee bodies or hive products). Moreover, these matrices do not contain just one contaminant, but a mixture of chemically different residues, which are ideally separated and determined at the same time in a single assay.

After separating the residues from the selected matrix, it can be useful or necessary to carry out clean-up procedures, since bee or bee product extracts usually contain interferents such as fats, pigments, and carbohydrates. In some cases, analysis of the analyte will require a concentration step to ensure that the analyte level is above the detection limit of the analytical method used.

The extraction and purification procedures applied depend on the composition of the matrix examined—either pollen, beebread, honey, wax, propolis, royal jelly, or the whole bee (Rossi et al. 2001; Fernández et al. 2001, 2002; Ghini et al. 2004). Gel permeation chromatography (GPC), liquid–liquid extraction (LLE), solid-phase (micro)extraction (SP(M)E), matrix solid phase dispersion (MSPD), supercritical fluid extraction (SFE), and ultrasonic extraction (UE) are the most commonly used techniques (Bargańska and Namieśnik 2010). Extract clean-up is often performed using separation columns filled with materials such as XAD-4, Florisil, C18, or graphite soot, by filtering the extract through Celite layers, and/or by adding coagulating solutions such as an ammonium chloride and orthophosphoric acid mixture. All of the analytical methods employed are multiresidual, and a QUEChERS technique is highly recommended for organic pollutants extracted from organic matrices, including pesticides.

Given that the extracts contain a large number of organic compounds with a wide range of chemical characteristics, both liquid chromatography (LC) and gas chromatography (GC) are employed to analyze them. LC is generally applied to examine thermolabile, polar, and low-volatility compounds, whereas GC can be used to determine thermostable and/or volatile compounds. Both of these chromatographic approaches are frequently coupled to mass spectrometry (MS), tandem mass spectrometry (MS/MS), or high-resolution mass spectrometry (HRMS) in order to achieve the maximum detection sensitivity (Kasiotis et al. 2014, 2018; Bargańska and Namieśnik 2010; Gómez-Ramos et al. 2019; Tong et al. 2018; Niell et al. 2017). However, other detection techniques can be employed for pesticide analysis. For example, we identified compounds that had been separated by GC using nonselective detectors such as nitrogen phosphorus (NPD), flame ionization (FID), and electron capture (ECD) detectors (Rossi et al. 2001). We also coupled an UV detector, or—after a postcolumn photochemical derivatization stage—a fluorescence detector to a HPLC instrument (Gil García et al. 2007).

LC-based separation can be coupled to mass spectrometry detection by various interfaces, such as electrospray ionization (ESI) (Kasiotis et al. 2018) or atmospheric pressure chemical ionization (APCI)—a soft ionization technique that yields quasimolecular ions and results in high detection sensitivity and information on molecular weight (Totti et al. 2006). GC is often coupled to mass spectrometry by a “hard” electron ionization (EI) interface.

The number of organic contaminants (not only pesticides) in the environment continues to rise each day, and new sample treatments (e.g., QuEChERS) are able to extract all of them, including unknown or untargeted pollutants such as metabolites of the targeted pollutants (Cotton et al. 2014; Kasiotis et al. 2018; Gómez-Ramos et al. 2016, 2019). Mass spectrometers operating in multiple reaction monitoring (MRM) mode cannot detect all of these compounds, so researchers prefer to use HRMS detection, which is performed in a full-scan mode such as time-of-flight (TOF), Fourier transform ion cyclotron resonance (FT-ICR), or Orbitrap analysis. These multiresidual methods make it possible to detect the presence of compounds that are banned or unauthorized for use in the region of interest, which might otherwise never be investigated (Malhat et al. 2015; Perugini et al. 2018).

Enzyme and monoclonal antibody-based immunoassays are also being developed. These methods strongly contrast with those described above, as they are very specific, sensitive, and rapid techniques for determining the presence of a specific target compound in a complex mixture of chemicals. Introducing chemiluminescent end-point detection into an immunoassay can greatly improve its detection limit. These



assays have been successfully used to detect a compound present at trace levels (Botchkareva et al. 2003; Mickova et al. 2005; Ivanov et al. 2008; Girotti et al. 2008, 2010; Soler et al. 2008).

Recently, Gillanders et al. (2019) developed a special detection method for explosive residues. The main hurdle to explosive detection is obtaining a sufficient amount of analyst for analysis, so the Authors placed strips containing a commercial fluoropolymer (AFLAS) in the entrances to the beehives. Explosive molecules collected by honeybees while flying are collected, absorbed, and concentrated by the strips as the bees enter their hives. Subsequent detection of the explosive occurs via the luminescence quenching response from the organic polymer Super Yellow (Gillanders et al. 2019).

## Conclusions

Environmental pollution is one of the main issues that must be addressed by contemporary society, and the global nature of this problem implies that methods of monitoring the quality of the environment should be suitable for application in regions with completely different environmental, infrastructural, social, and economic conditions.

Biomonitoring utilizes organisms that are sensitive, ubiquitous, and stable in the territory of interest to obtain accurate information on the pollutants present in the territory as well as their biotoxic effects. Honeybees have been shown to satisfy all these requirements.

Wild and domestically raised honeybees are widespread in the Mediterranean area. The chance to collect and compare homogeneous data obtained from large territories using the same sampling technique makes it possible to derive unexpected and very useful information for the integrated management of environmental aspects at scales larger than individual countries. Moreover, data obtained by analyzing bees and their products can help beekeepers to preserve bee populations by avoiding heavily contaminated areas. Such data can also be useful when attempting to establish environmental limits on the concentrations of contaminants that are toxic to humans or bees (Sgolastra et al. 2020).

Finally, one of the key aspects of sustainable development is the active participation of citizens in actions to tackle environmental issues. This is particularly true of young people, for whom we must conceive and develop appropriate educational programs. Committed as we are to helping to educate the public in environmental issues, we have prepared an online open-access course in multiple languages (Italian, Spanish, and English) as a means to pass on our knowledge of the application of honeybees in environmental protection activities (<http://openlearning.unibo.it/?lang=en>).

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## Compliance with ethical standards

**Conflicts of interest** All author(s) declare that they have no conflict of interest.

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