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Research Article

Influence of Geographic Separation Between Urban Centers and Microplastic Burden on Bees (*Apis mellifera*)

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Abstract

Plastic, a product of industrialization, has become an integral part of human life since its inception. Its extensive usage has led to a drastic increase in production, resulting in the widespread accumulation of plastic waste at various levels. Improper disposal practices have compounded this issue, transforming plastic waste into a significant environmental concern. Factors such as the sheer volume of plastic generated, inadequate management, persistent accumulation, and fragmentation exacerbate this problem. Of particular concern is the prevalence of microplastics, tiny particles measuring less than 5mm, which have permeated various natural ecosystems and have been detected within living organisms. For example, bees, due to their foraging activities, inadvertently carry microplastics and associated contaminants on their bodies. This study aims to investigate the presence of microplastics in bees (*Apis mellifera*), utilizing them as indicators of microplastic pollution. Additionally, it seeks to determine whether there is a correlation between proximity to urban centers and the abundance of microplastics in bees sampled from selected apiaries. Employing fluorescence microscopy and FTIR Spectroscopy, the study analyzed a total of 54 samples collected from 9 participating apiaries within the designated area. Statistical analyses revealed that Gualaceo is not immune to

microplastic contamination, with fibers and synthetic fragments detected in bee specimens. Furthermore, the study found an inverse relationship between distance from urban centers and the concentration of microplastics within beehives. To advance knowledge in this field, future research could explore the extent to which microplastics are integrated into bee products and their potential impact on living organisms. These findings underscore the urgent need for a shift in attitudes and actions at both individual and institutional levels, emphasizing the importance of proactive engagement in formulating and implementing effective environmental policies and engineering solutions.

Keywords

Apiaries, Bioindicator, Micro-plastics, Fluorescence microscopy, FTIR spectroscopy.

Introduction

The increasing production of plastics and their careless disposal are causing significant pollution in various ecosystems, posing a major challenge for humanity. Microplastics, fragments smaller than 5 mm, result from the fragmentation and dispersal of plastic waste in the environment, contaminating soil, water, air, and entering living organisms through ingestion, respiration, and electrostatic adhesion.

These microplastics, considered a public health issue, have been found in human blood and everyday products. To assess their impact, laboratory scientific procedures are employed, using bioindicators such as bees (*Apis mellifera*), which are excellent indicators of pollution in ecosystems due to their extensive foraging and interaction with various resources.

Microplastics pose an increasing threat to bees, with acute, chronic, and sublethal effects. Among the acute effects, it has been observed that accidental ingestion of microplastics can obstruct their digestive system, reduce their nutrient absorption capacity, and, in severe cases, cause death. Microplastic particles can lodge in the bees' respiratory tracts, causing asphyxiation, and sharp fragments can physically damage their wings, legs, and other body parts, hindering their movement and foraging ability Fernandez (2022).

In the long term, exposure to microplastics can affect bees' reproduction, reducing the number of eggs they lay and the viability of their offspring, and weakening their immune system, making them more susceptible to diseases and parasites. Additionally, bees exposed to microplastics have been observed to exhibit altered behaviors, such as difficulty finding their way back to the hive and reduced communication capacity. Sublethal effects are also concerning, as microplastics can affect bees' ability to pollinate flowers, negatively impacting crop production and ecosystem health. Furthermore, microplastics can bioaccumulate in the food chain, meaning that bees that ingest them

can be consumed by other animals, including humans, with potentially harmful consequences Vivarelli (2024).

The international research on microplastics in bees and their environment reveals significant concerns. A study by Al Naggar et al. (2021) addresses the widespread presence of microplastics in bees and honey globally, highlighting the need for further investigation into the potential health risks for these pollinators. Edo et al. (2021) demonstrate the presence of microplastics in bees collected in Denmark, identifying various types of MPs and noting high levels in urban, suburban, and rural areas. Deng et al. (2021) investigate the susceptibility of bees to viral infections due to the ingestion of polystyrene microplastics, emphasizing the bioaccumulation of MPs and their effects on intestinal microbiota. Other studies, such as Molina-Castro (2021) and González-Pleiter et al. (2021), explore detection methods and the presence of microplastics in marine organisms and the atmosphere, respectively. Additionally, studies in Colombia underscore the presence of microplastics on its coasts, linking them to factors like river influence, wastewater discharges, and human activities. Molina-Castro (2021) evaluates pollution on the South Pacific coast, highlighting inadequate solid waste management as the main cause and emphasizing the increased plastic waste during the COVID-19 pandemic, with alarming projections for 2030. Collectively, these studies emphasize the urgent need to address the risks associated with microplastics in the ecosystem and the importance of adopting preventive measures.

The studies conducted in Ecuador address the presence and impact of microplastics (MPs) in various environmental and consumable sources. Diaz-Basantes et al. (2020) investigated the occurrence of MPs in widely consumed beverages such as milk, soft drinks, honey, and beer. They found that at least 12% of the analyzed samples contained synthetic fragments and fibers, predominantly composed of polyethylene, polypropylene, and polyacrylamide. The study suggests that honey collected from densely populated areas may have higher MP concentrations. Another study made by Capparelli et al. (2021) was focused on assessing microplastic pollution in the coastal waters of the province of Esmeraldas, Ecuador. The research covered fourteen coastal areas with varying urbanization levels and ten water bodies. The results showed that coastal waters had significantly higher quantities of MPs compared to river waters, with transparent fibers and colored fragments being the most prevalent. Both studies highlight the widespread presence of microplastics in Ecuador's environment, raising concerns about the potential impact on human health and ecosystems. These findings emphasize the importance of continued research and environmental management strategies to address the growing issue of microplastic pollution.

This study builds on the work by Orellana (2023) which focuses on the characteristic of bees as bioindicators to investigate the presence and quantity of microplastics in their bodies in relation to the distance from human settlements. The study area is the Gualaceo canton, Azuay province, in southern Ecuador, where similar studies are lacking. The aim is to characterize microplastics, determine their quantity in bees, and analyze if there is a relationship with the distance to urban centers. The methodology

includes literature review, collection of bee samples, and their treatment with fluorescence inverted microscopy, FTIR spectroscopy, and photographic devices.

The study aims to provide additional knowledge, raise awareness in society about the danger of microplastics in ecosystems, and encourage environmentally friendly public policies to reduce negative impacts.

Materials and Methods

Study Area

The study is conducted in the peri-urban area of Gualaceo Canton, situated around the urban area of Gualaceo city, in Azuay Province, southern Ecuador. The areas under study include locations where beekeepers have their apiaries: to the north, La Isla (Bullcay), Racacay, Bullcay (Stadium), and Negas; to the south, Cochapamba and Nallig; to the east, Yabrum and Quimzhi; to the west, Guazhalan. These areas are transitional between urban and rural, characterized by continuous changes in land use for productive and residential activities, Table 1 shows the geographical coordinates of the sampled apiaries and Fig. 1 a map with their locations. They feature incomplete infrastructure and low building density, with extensive open areas dedicated to agricultural and/or livestock production.

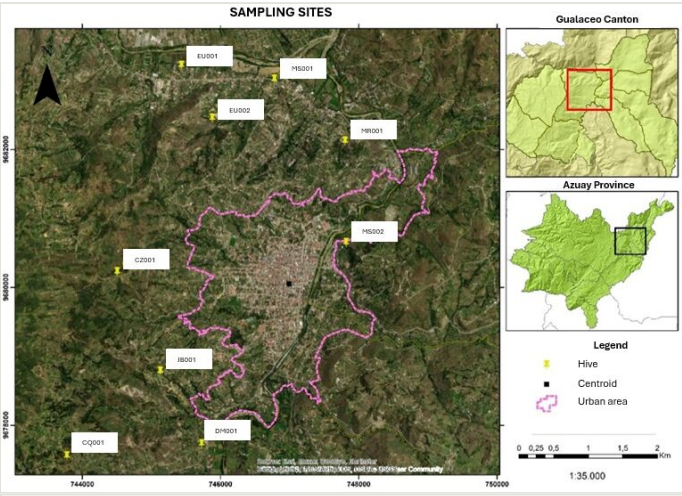

Figure 1.

Locations of sampled apiaries.

The urban area of Gualaceo covers an area of 6.58 square kilometers, with a population of 13981 inhabitants in the urban core and 7462 individuals in the periphery, according to data from the VII Population and VI Housing Census 2010 conducted by the National Institute of Statistics and Census (INEC). This urban area stands out as the most populous in the region. Geographically, it is located in the Santa Bárbara river valley.

The analysis phase of the research was conducted during the penultimate quarter of the year 2022, with the voluntary collaboration of beekeepers from the study area, who provided essential information for the research and participated in sample collection.

Table 1.
Geographical coordinates of sampled beehives (Orellana 2023).

Apiary code	# hive	Sector	Geographical coordinates (WGS 84 / UTM zone 17S)		Distance to the urban center (Centroid)
			X	Y	
MS001	3	Bullcay	746780	9683020	2.98 km
MR001	3	Negas	747805	9682123	2.22 km
MS002	3	Guazhalan	747819	9680651	1.02 km
EU001	3	La Isla	745437	9683223	3.53 km
EU002	3	Racacay	745883	9682457	2.64 km
JB001	3	Quimzhi	745131	9678784	2.3 km
CQ001	3	Cochapamba	743780	9677557	4.06 km
CZ001	3	Yabrum	744507	9680227	2.49 km
DM001	3	Nallig	745728	9677731	2.65 km
Total	27				

Protocols Applied in Research

Prior to implementing the methodology, a review of methodologies utilized in comparable research was conducted. The most appropriate techniques for conducting this study were gathered, and tailored to suit the inventory of the Life Sciences laboratories at the Polytechnic University Salesiana Campus in Cuenca. The process is segmented into the subsequent stages.

Sample collection

To establish the sample collection protocol in the different accessible apiaries, similar research on the participation of bees as active collectors of microplastics was considered (Edo et al. 2021). After coordinating with the beekeepers, each apiary was accessed with the appropriate safety equipment and the necessary materials for the collection, storage and conservation of the samples. For approximately one month, 50 dead worker bees that were around each hive were collected, which were then placed in 130 ml glass jars with cork lids, properly labeled and sealed. Three hives per apiary were analyzed. These samples, organized by apiary, were immediately placed in zip-lock bags and refrigerated in a cooler to maintain their integrity and prevent decomposition. Subsequently, the samples were frozen without adding any solution until they were transported to the Life

Sciences laboratory of the Salesiana Polytechnic University of Cuenca. This ensured that the samples were ready for corresponding analysis. This process was repeated after a period of 15 days in each of the apiaries selected for initial sampling. Suppl. material 1 shows the sampling design, with its respective dates per apiary and per hive.

Extraction of microplastic from the study environmental matrix

- **Sample processing and removal of microplastic particles**

Once the samples arrive at the laboratory, they are thawed at room temperature. Then, the individuals are transferred to a 250 ml glass precipitation beaker for counting, to which a 50% solution composed of 50 ml of ethanol and 150 ml of Milli-Q water is added, gently stirring with a glass rod for 15 minutes to release microplastic particles and other substances adhered to the bodies of the bees. This process is carried out in a laminar flow hood to prevent any cross-contamination. The Milli-Q system is a modern water purification system that dispenses high-quality ultrapure water (Type I) and purified water (Type II).

- **First leak**

After 15 minutes of sample agitation, filtration is performed using filter paper with a porosity of 11µm in the sample vacuum filtration kit. With the remaining 50% of the ethanol and MilliQ water solution, the bodies of the bees are rinsed on the same filter to recover any possible particles or materials that may remain attached to them, taking care to avoid disintegrating their bodies. The filtered solution, corresponding to an approximate volume of 200 ml, is reserved for the digestion or removal of organic matter stage. It should be noted that only the filtration process is carried out outside the laminar flow hood, always ensuring that the samples are covered with aluminum foil to prevent cross-contamination.

- **Oxidation or digestion of the sample**

The solution resulting from the first filtration of the sample, which is in the Kitasato, is transferred to a 250 ml precipitation flask. Then 20 ml of 33% hydrogen peroxide (H_2O_2), which is 10% of the sample, are added with the aim of eliminating and detaching any organic matter present in the sample and obtaining only the microplastic particles. Once the hydrogen peroxide is added, the sample is placed in the oven at a temperature of 60°C for 24 hours, conditions applied in another similar study (Edo et al. 2021).

- **Microfiltration and separation of microplastic particles.**

Upon completing the designated time for the removal of any organic matter present in the sample, the separation of microplastic particles from the treated solution is carried out through filtration using the vacuum filtration system and a cellulose nitrate membrane filter with a diameter of 47 mm and a porosity of 0.45 µm. Once this stage is completed, the filter is carefully removed, placed in a glass petri dish, and covered with aluminum

foil, ensuring the proper coding of each sample. Subsequently, it is left to dry at room temperature for 24 hours.

Staining technique

During this stage, a staining solution is prepared using absolute methanol and Nile red, with a concentration of 1ppm, which is then transferred to a spray dispenser. To avoid light sensitivity, the dispenser is covered with aluminum foil and stored in a dry, dark place at room temperature throughout the analysis.

After 24 hours have passed, a layer of Nile red dye is applied to the filter resulting from the previously mentioned filtration process. Subsequently, the filter is allowed to dry completely before proceeding with the observation of microplastics under the microscope. It is important to note that inadequate drying could affect the intensity of fluorescence when visualizing microplastics under a fluorescence microscope (Prata et al. 2020).

The application of nile red dye to the filter containing microplastics improves the efficiency and accuracy in the identification and quantification of microplastics due to its solvatochromic property. This method is cost-effective, easy to use, and allows rapid identification of microplastics (Maes et al. 2017Shruti et al. 2022). It also facilitates the differentiation between plastic particles and other particles simultaneously, due to its affinity for hydrophobic particles (Patchaiyappan et al. 2021).

Characterization by observation of microplastics

For the visualization of microplastic particles, the Nile red-stained filter, which has already been dehydrated, is placed on a glass slide and observed under a Nikon Eclipse Ti2 fluorescence inverted microscope. The software (NIS Elements BR) is used to guide the process carefully. To observe fluorescence, it is set to the green filter of the equipment, with an excitation wavelength of 430-490 nm and an emission wavelength of 510-560 nm (Stanton et al. 2019). During observation, a visual scan is performed from left to right to detect microplastic fragments or fibers emitting fluorescence, facilitating their identification. The shape, size (length or area), quantity, and location of particles on the filter are then determined. Before observation, the filter is cautiously divided into four quadrants to facilitate visualization.

Analysis by Fourier Transform Infrared Spectroscopy (FTIR) of microplastics (MPs)

After the observation and location of the particles of interest in the filters in the previous stage, we move on to the analysis of the main components of all identified particles. These are subsequently analyzed using the FT-IR Spectrometer, Nicolet™ iS™ 10 model, from Thermo Scientific, with the help of an integrated software called OMNIC. The analytical methodology applied in this instrument is based on Fourier transform infrared spectroscopy (FTIR) with the attenuated total reflectance (ATR) technique using diamond.

At least three scans are performed for each particle present in the sample. The applied wavelength covers a range of 400 to 4000 cm^-1, corresponding to the mid-infrared. This procedure is controlled and evaluated through the OMNIC software, which allows determining the spectra of each particle, comparing them with the reference library data in the program. Similarities were found between the spectra of the reference library and those of the sample, which correlates with the behaviors of synthetic polymers in previous studies as findings by Song et al. (2015). Finally, once the spectra of the polymers have been identified, statistical analysis will be performed to examine and interpret the data using statistical tools. This will allow verifying if there is any relationship between the distance of urban centers and the quantity of microplastics in the beehives of beekeepers in the peri-urban area of the Gualaceo canton.

Statistical analysis

ANOVA was used to test for differences in microplastic levels among apiaries, and a *Residual Normality Test* was conducted to confirm the normality of residuals, a crucial assumption for *ANOVA*'s validity. Given that initial residuals were non-normal, a *Box-Cox Transformation* was applied to identify the best normalization transformation. This led to the use of a *Logarithmic Transformation*, which successfully normalized the residuals. Post-transformation, the *ANOVA* model was re-adjusted with the transformed dependent variable, and the normality of the residuals was checked, confirming the validity of the model. Subsequently, the average amount of microplastics per 50 bees in each hive was calculated, consistently applying the previously used logarithmic transformation. Finally, a *Correlation Analysis* was then employed to understand the relationship between microplastic levels and distance, providing a clear, statistically sound understanding of how urban proximity impacts microplastic contamination in bees.

To enhance the accuracy and understanding of the results, Excel and R were used, facilitating the generation of tables, graphs, and the execution of relevant hypothesis tests for this specific study context.

Results

Visual characterisation of microplastics by their shape

Two different forms or structures of polymers were identified. The fiber, characterized by its length as shown in Fig. 2, and the fragment, characterized by area or diameter as shown in Fig. 3. This characterization is done through both simple microscopy observation and fluorescence microscopy. The illumination projection in both the fiber and the fragment confirms their presence.

Abundance of microplastics in the bees

The examination of samples from selected beehives revealed the presence of microplastics (MP) in both fragment and fiber forms. The total quantity of these synthetic microparticles was determined for each analyzed apiary, showing variations in observed MPs among samples as shown in Table 2. A total of 1269 polymeric particles were observed in the study area, including 583 fragments and 686 fibers. The overall average of MPs per 50 bees (each sample) is 24 MPs/50 bees. The MSG001 beehive reports the highest average MPs at 57 MPs/50 bees, while the CQ001 beehive has the lowest at 10 MPs/50 bees.

Table 2.
Number of fibers and fragments counted in each sample (Orellana 2023).

	Nº of hive	H1 (1st sampling)	H2 (1st sampling)	H3 (1st sampling)	H1 (2nd sampling)	H2 (2nd sampling)	H3 (2nd sampling)	Average
JB001	**Fragment**	21	34	21	33	52	7	28
	Fibers	18	16	29	31	26	19	23
	Concentration (MPs/sample)	39	50	50	64	78	26	**51**
MR001	**Fragment**	0	0	9	4	6	1	5
	Fibers	12	5	12	11	5	6	9
	Concentration (MPs/sample)	12	5	21	15	11	7	**12**
MS001	**Fragment**	20	15	19	18	2	1	13
	Fibers	27	20	7	12	4	8	13
	Concentration (MPs/sample)	47	35	26	30	6	9	**26**
CQ001	**Fragment**	3	8	12	2	1	0	4
	Fibers	5	10	8	4	6	3	6
	Concentration (MPs/sample)	8	18	20	6	7	3	**10**
CZ001	**Fragment**	10	2	1	4	4	4	4
	Fibers	15	6	9	9	6	7	9
	Concentration (MPs/sample)	25	8	10	13	10	11	**13**
MS002	**Fragment**	27	36	30	38	39	14	31

	Nº of hive	H1 (1st sampling)	H2 (1st sampling)	H3 (1st sampling)	H1 (2nd sampling)	H2 (2nd sampling)	H3 (2nd sampling)	Average
	Fibers	25	30	23	39	22	18	26
	Concentration (MPs/sample)	52	66	53	77	61	32	**57**
DM001	**Fragment**	4	11	0	15	8	9	8
	Fibers	4	13	13	3	16	15	11
	Concentration (MPs/sample)	8	24	13	18	24	24	**19**
EU001	**Fragment**	4	7	1	4	2	2	3
	Fibers	8	15	6	7	3	12	9
	Concentration (MPs/sample)	12	22	7	11	5	14	**12**
EU002	**Fragment**	2	10	5	0	0	1	3
	Fibers	9	12	7	17	3	10	10
	Concentration (MPs/sample)	11	22	12	17	3	11	**13**
		Total	**Average**	**Percentage**				
Fragment		583	11	45.94%				
Fibers		686	13	54.06%				
Concentration (MPs/sample)		1269	**24**	100%				

Note: Each sample corresponds to 50 bees analyzed. / H1-H3 are the number of hives sampled per apiary. / Codes as JB001 or MR001 refer to the code of the apiary.

Fig. 4 provides the average number of MPs found in the studied beehives, categorized by each participating apiary. The figure illustrates averages for synthetic fragments, synthetic fibers, and their total concentration. Beehives with JB and MSG codes have the highest identified MPs/50 bees on average, while beehive CQ has a lower average. Furthermore, the range of registered diameters for synthetic fragments is from 5.63 µm ± 1834.69 µm, and for synthetic fibers, the range of lengths is from 6.65 µm ± 4980.57 µm (minimum and maximum).

In the subsequent analysis, data from samples collected in the nine apiaries were evaluated using descriptive statistics. Apiary CQ001 exhibits a lower mean of MPs/50 bees at 10.33, while MS002 has the highest mean at 56.5 MPs/50 bees. Apiaries MR001, EUI001, and EU002 show a similar central tendency with a value of 11.5, while the rest differ. Standard deviation values indicate greater dispersion. Regarding the mode, some

apiaries have a frequent value, while others lack a mode due to the absence of data periodicity.

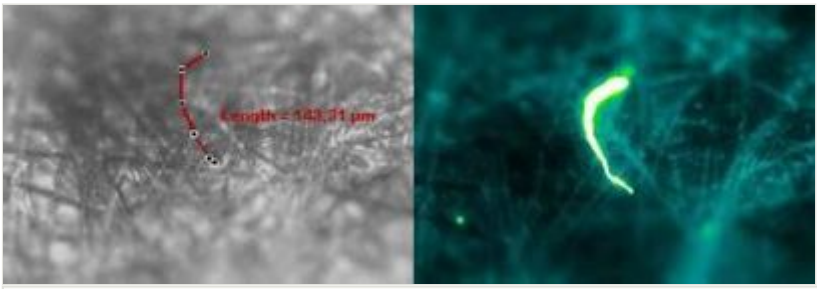

Figure 2.

A. Micrometric synthetic fiber with its shape and length; B. Synthetic fiber with fluorescence.

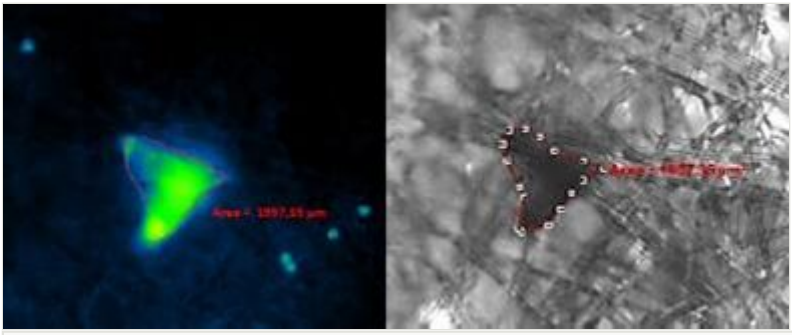

Figure 3.

A. Irregular micrometric synthetic fragment, in its shape and length; B. Synthetic fragment with fluorescence.

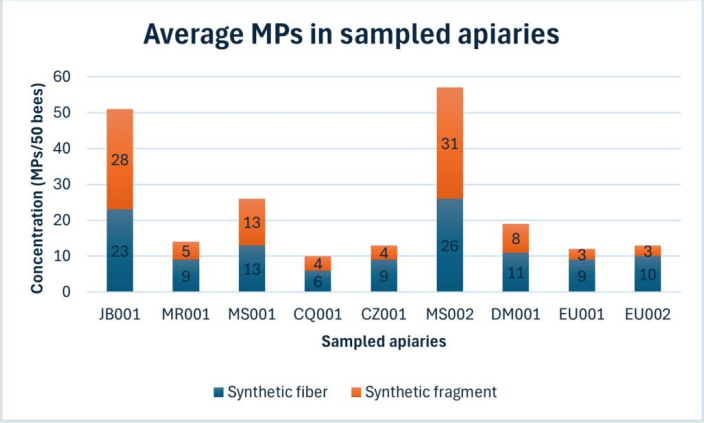

Figure 4.

Average number of microplastics in the form of fiber and fragment observed during sampling (MPs/50 bees).

Composition of microplastics in bees based on Fourier Transform Infrared spectrophotometer

The observation and identification of particles on the filters using the inverted fluorescence microscope, marked for measurement in the FTIR, and supported by the OMINIC program, allowed for the verification and identification of the composition of particles that could be detected by the spectrophotometer in the samples. The spectra were found to be similar to, or correspond to, those indicated in the reference library, which includes HR Hummel Polymer and Additives, HR Polymer Additives and Plasticizers, HR Specta Polymers and Plasticizers by ATR, HR Specta Polymers and Plasticizers by, and Hummel Polymer Sample Library. The study focuses on identifying the presence of synthetic polymers without characterizing them by bandwidth ranges. An unforeseen misconfiguration of the OMNIC Spectra software complicated the verification process but did not invalidate the results. The percentage of coincidence between the sample and reference spectra varied, with the study choosing percentages higher than 70% for greater precision. However, it is acknowledged that coincident values can reach 47.50%, considered a good result (Qiu et al. 2015).

Fig. 5, Fig. 6 and Fig. 7 show the analysis of the identification of some polymers in three samples from different apiaries, based on their composition by comparing spectra of the sample particle as provided by the FTIR. Additionally, the matching value is visible in the images.

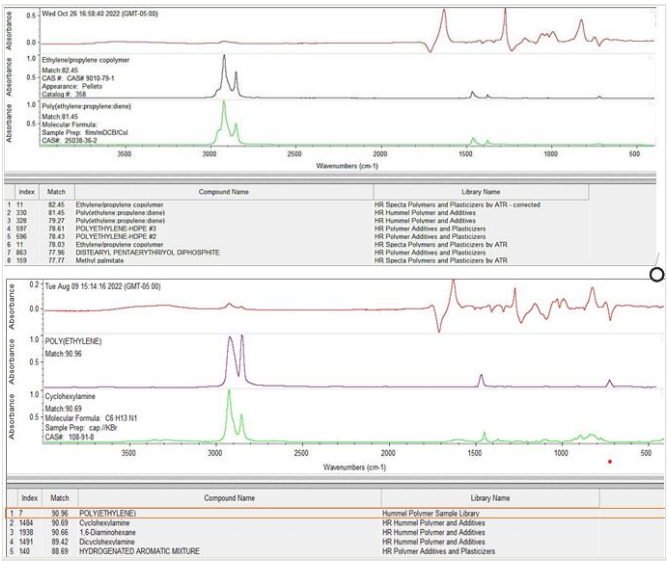

Figure 5.

The spectra of two different fibers from a sample from apiary JB001 (red) and the reference library-FTIR (blue and green) indicate which polymer corresponds to its composition and its matching value. Poly(ethylene: propylene: diene); Polyethylene (PE).

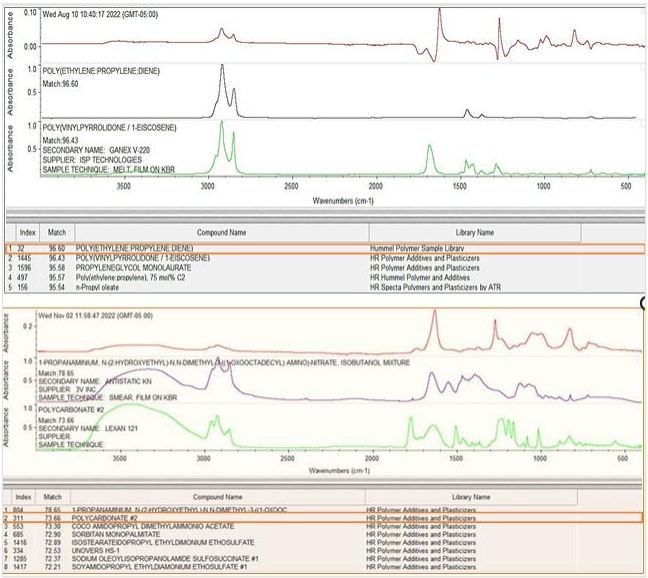

Figure 6.

The spectra of two different fibers from a sample from apiary MS001 (red) and the reference library (blue and green) indicate which polymer corresponds to its composition and its match value. Poly(ethylene-propylene-diene) (EPDM); Polycarbonate (PC).

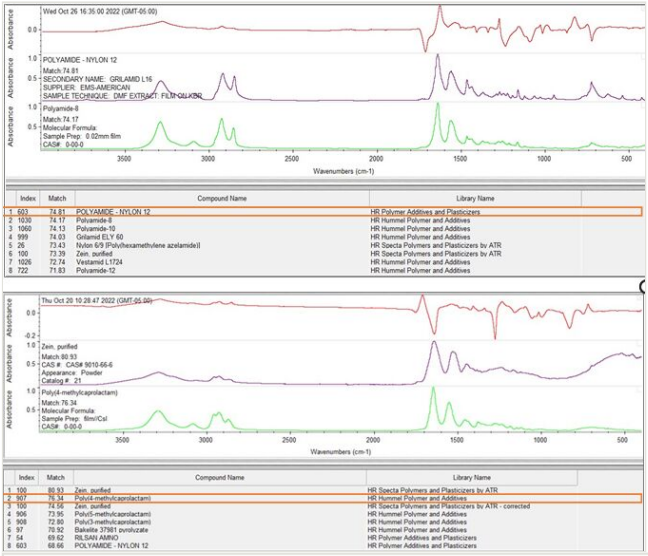

Figure 7.

The spectra of two different fibers from a sample from apiary CZ001 (red) and the reference library (blue, green) indicate which polymer corresponds to its composition and its match value. Polyamide-Nylon 12 Polymer, PA; Poly(4-methylcaprolactam).

Table 3 provides insights into the types of polymers identified by composition in each sampled beehive, with codes corresponding to collaborating beekeepers. A total of 341 particles were identified across 23 polymers. The presence of PET was notable in almost all sites, except in the EUI hive, while poly(4-methylcaprolactam) was absent in MR and MS002 hives. Nylon 12 was found in 7 sites but not in MS002 and EUR hives, and polycarbonates were present in 6 hives, excluding MR and DM.

Table 3.

Type of polymers by their composition and quantity recorded in all the bees collected across all samples in the apiaries (Orellana 2023).

Type of Polymers by their composition	**Apiaries sampled/Amount of polymers recorded**									**Total**	%
	JB 001	MR 001	MS 001	CQ 001	CZ 001	MS 002	DM 001	EU 001	EU 002		
Polyethylene (PE)	27		9	1		2	1			40	11.73
Polyethylene -HDPE#1	5		1							6	1.76
HD polyethylene	4	2								6	1.76
Polyethylene propylene diene (EPDM)	28		3			1	1	1		34	9.97
Polyethylene propylene	1				1					2	0.59
Ethylene-propylene copolymer (EPM)	3						2			5	1.47
Nylon 6,9	1			1			1			3	0.88
Nylon 6, 3	2									2	0.59
Nylon 12	2		2		1					5	1.47
Polyamide-nylon 12	4		24	1	1		7	3		40	11.73
Polyamide 8							13	2		15	4.40
Polyamide 10							3			3	0.88
Polyethylene terephthalate (PET)	1	3	1	2	1	1	1		1	11	3.23
Polytetrafluoroethylene	7	12	2			1				22	6.45
Polyester	1									1	0.29
Polycarbonate #2	2		2	4	1	4		2		15	4.40
Poly(4-methylcaprolactam)	4		18	6	15		2	26	15	86	25.22
Poly(5-methylcaprolactam)	1			3	1			3		8	2.35
Polypropylene	1				1					2	0.59
Poly(butadiene acrylonitrile)		8								8	2.35
Bakelite 37981						2		2		4	1.17
*Zeina	9				1					10	2.93
*Silk						13				13	3.81
	103	25	62	18	23	24	31	39	16	341	100.00

Note: The sign (*)indicates that these two registered polymers are of natural origin. / Codes as JB001 or MR001 refer to the apiaries sampled.

The distribution of polymer quantities by typology is further detailed in Table 3, with percentages relative to the total types found (341 particles). Poly(4-methylcaprolactam) leads with 25.29%, followed by Nylon 12 and Polyethylene (PE) at 11.73% each. Ethylene Propylene Diene Monomer (EPDM) accounts for 9.9%, Polytetrafluoroethylene (PTFE) at 6.45%, Polyamide 8 and polycarbonate at 4.4% each, and Polyethylene Terephthalate (PET) at 3.23%. These prominent polymers collectively contribute to 73.9%, while the remaining polymers constitute 26.10% of the total.

The OMNIC software plays a crucial role in eliminating initially recorded false positives during fluorescence microscope visualization, ensuring accurate identification of synthetic polymer types. It verifies the recorded spectrum against the library spectrum, facilitating the collection, classification, and quantification of polymers in samples from various beehives. However, fluorescence microscopy is highly sensitive to small particles, allowing the detection of a larger number of microplastics. In contrast, FTIR, while excellent for chemical characterization, may have limitations in detecting very small particles due to its lower spatial resolution and the efficiency of infrared light dispersion.

Statistical differences in the number of microplastics among apiaries

An ANOVA was conducted to investigate the differences in microplastic levels among apiaries. The analysis revealed that the model residuals did not follow a normal distribution. In light of this observation, the application of a Box-Cox transformation was recommended to normalize the residuals and meet the assumptions of the analysis (normality and homoscedasticity). After performing the logarithmic transformation, the model is rerun using the transformed dependent variable; this involves fitting the ANOVA model with the logarithm of the amount of microplastics as the response variable. The residuals' normality and the variance constancy (homoscedasticity) are then checked.

The result of this test confirmed that both of the assumptions are satisfied post-transformation; therefore, the model is considered valid, with the log-transformed amount of microplastics as the response variable.

To illustrate the distribution of the logarithmic amount of microplastics (logC_MPs) in 9 different apiaries (marked 1 to 9 on the x-axis), Fig. 8 was created. The black lines within the violins represent the quartiles of the distribution, while the red dots highlight the logarithmic mean of the amount of microplastics. Overall, the diagrams reveal variations in the distributions of logC_MPs among the apiaries, indicating differences in microplastic loads that may be influenced by environmental or management factors specific to each apiary.

The distribution of logC_MPs varies significantly between apiaries. Apiaries such as JB001 and MS002 have higher means (logC_MPs = 3.88 and 4.01, respectively), indicating higher microplastic contamination. While, apiaries such as MR001 and CZ001

have lower means (logC_MPs = 2.37 and 2.13, respectively), suggesting lower amounts of microplastics.

A Welch ANOVA analysis was performed and found to be significant (F_{Welch}(8, 18.64) = 13.67, p = 2.51e-06), indicating significant differences between apiaries. Moreover, Games-Howell post hoc tests showed significant differences between several pairs of apiaries, especially between apiary MS002 and apiaries MR001, MS001, CZ001, DM001, EU001, and EU002 (adj. p_FDR ≤ 0.04).

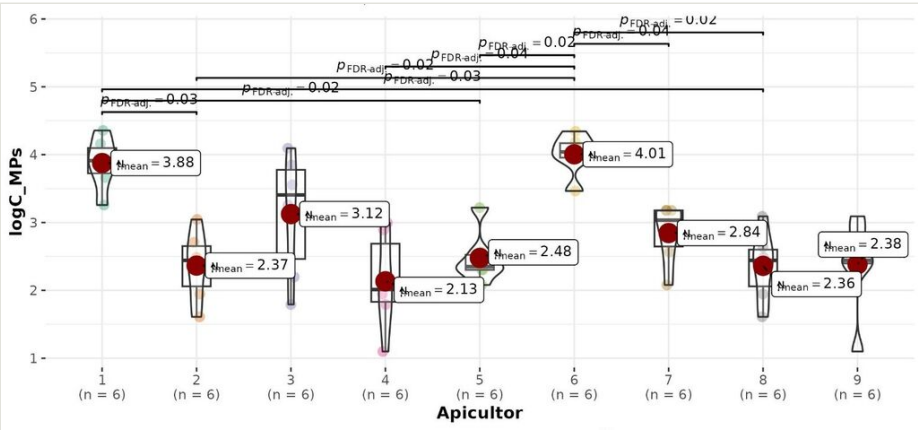

Figure 8.

Violin plot showing the distribution and comparison of the logarithmic amount of MPs / 50 bees (logC_MPs) in 9 different apiaries (1:JB001, 2:MR001, 3:MS001, 4:CQ001, 5:CZ001, 6:MS002, 7:DM001, 8:EU001, 9:EU002).

The relationship between distance to urban centers and microplastics in bees

Finally, this research, which is not only descriptive but also inferential, aims to establish a relationship between the distance to the urban center of Gualaceo and the amount of microplastics found in the bodies of bees (*Apis mellifera*) within hives located in the peri-urban area of the Gualaceo canton. The average amount of MPs per 50 bees is calculated for each hive across the nine apiaries. Since a logarithmic transformation was previously applied to the data for normalization purposes, the same transformation is consistently applied to the calculated averages to maintain consistency in the analysis; this means that the log-transformed average amount of microplastics per hive is used in the subsequent analysis to explore the relationship with the distance to the urban center.

To facilitate understanding of the relationship between both variables (distance to the urban center of Gualaceo and the logarithm of the amount of microplastics detected in bees), a scatter diagram (green plot) was created (Fig. 9). The plot illustrates a statistically significant negative correlation between the variables. This suggests that bees located farther from the urban center of Gualaceo tend to have lower amounts of

microplastics, highlighting a potential impact of urban proximity on microplastic contamination in bees.

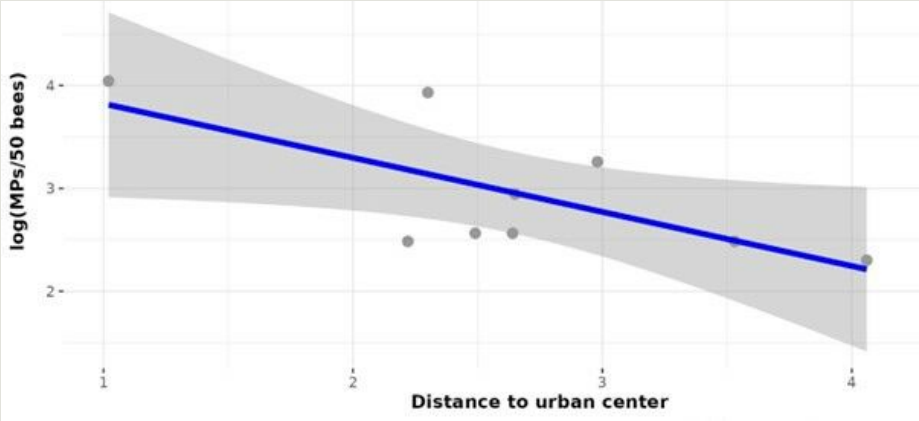

Figure 9.

Scatter plot with a linear regression model between the distance to the urban center of Gualaceo and the amount of microplastics detected log(MPs/50bees).

The blue line represents the fitted linear regression model that follows the equation $MP_log = 4.34 - 0.52 * (distance)$, and the shaded gray area represents the 95% confidence interval for the regression line; the coefficients and extra information are shown in Table 4 . The confidence interval narrows around the regression line, indicating the precision of the estimated relationship.

Table 4.

Coefficients of the linear regression

Coefficients				
	Estimate Std.	**Error**	**t value**	**Pr (>t)**
Intercept	4.3484	0.5784	7.519	0.000135 ***
Dist (slope)	-0.5256	0.2085	-2.521	0.039741 *
R^2	0.4759			
F-statistic	6.356		**p-value**	0.03974

The linear regression analysis provided insightful results regarding the relationship between the amount of microplastics in bees and the distance from urban centers. The significance test (F statistic) produced a p-value of 0.039, which is less than the conventional threshold of 0.05. This indicates that the model significantly explains the variability in the data, affirming that the observed trend is statistically significant.

The coefficient of determination (R^2) was found to be 0.4759, suggesting that approximately 47.6% of the variability in microplastic levels in bees can be explained by the distance from urban centers. While this R^2 value indicates a moderate correlation, it is important to note that the relatively small sample size of nine apiaries may contribute to this level of explained variance. A larger sample size could potentially provide a more robust estimate of the relationship.

Furthermore, the regression slope was calculated to be -0.5256. This negative slope indicates a downward trend, implying that as the distance from urban centers increases, the amount of microplastics in bees tends to decrease. This negative trend aligns with the hypothesis that urban centers, being sources of pollution, have a higher concentration of microplastics, which diminishes with distance. On the other hand, the t-statistic of -2.521 indicates that the slope of the regression line is significantly different from zero, supporting the presence of a negative correlation.

Discussion

In the examination of 1269 fragments and fibers initially identified based on appearance and fluorescence, caution was exercised to discard false positives, aligning with the recommendations of Nalbone et al. (2021). Identifying microplastic particles, particularly smaller ones, required meticulous attention due to potential equipment failures or contamination, echoing Stanton et al. (2019). The application of Nile Red staining was confirmed as a valid technique, despite its potential for overestimation, proving accessible and effective in differentiating microplastics, as affirmed by Stanton et al. (2019). The predominant microplastics in all samples were irregular fibers and fragments, consistent with study objectives and findings by Edo et al. (2021) and Deng et al. (2021).

The abundance of microplastics showed 54.06% as fibers and 45.94% as fragments, differing from Edo et al. (2021). Measurement criteria for microplastics ranged from 1µm to 5mm for both fibers and fragments, in line with Frias and Nash (2019) and Castañeta et al. (2020). Synthetic polymers identified included Polyamides, Polyethylene, Ethylene Propylene Diene Monomer, Polytetrafluoroethylene, Polycarbonate, and Polyethylene Terephthalate, aligning with Deng et al. (2021) and Diaz-Basantes et al. (2020).

In evaluating the microplastics, both fluorescence microscopy and FTIR were employed to obtain a comprehensive view of the sample. Fluorescence microscopy enabled highly sensitive detection, revealing a higher quantity of microplastics, including very small fragments. This aligns with the technique's high sensitivity to fluorescent particles. However, FTIR, while effective in the chemical characterization of microplastics, showed lower detection in comparison, which may be attributed to its lower spatial resolution and the efficiency of infrared light dispersion, which can limit the detection of extremely small particles. This discrepancy highlights the importance of using complementary techniques for a more accurate assessment of the presence and characteristics of microplastics in the samples.

The study employed a descriptive correlational methodology, validating hypotheses and confirming an inverse relationship between microplastics in bees and the distance from urban centers. Statistical analysis supported this, emphasizing the need for continued investigation into microplastic pollution sources in beehives, considering environmental factors and human activities.

Conclusions

After a meticulous analysis of the data collected from peri-urban beehives in the Gualaceo canton, the extended presence of microplastics in honeybees (*Apis mellifera*) is conclusively confirmed, revealing an alarming reality with global repercussions. These findings not only corroborate the increasing concern regarding microplastic pollution but also underscore the urgent need for concerted action at local, national, and international levels to address this multifaceted issue.

The identification of a variety of plastic types, ranging from polyethylene to polyethylene terephthalate, in bee bodies, coupled with their geographic dispersion, suggests widespread and systemic pollution affecting ecosystems as a whole. These results emphasize the importance of raising public awareness about the impacts of microplastic pollution and the necessity of implementing effective waste management policies and environmental protection measures.

Furthermore, the observation that the quantity of microplastics decreases as the distance from urban centers increases highlights the interconnectedness between human activity and environmental pollution, emphasizing the importance of adopting mitigation and prevention measures in urban and peri-urban areas.

Ultimately, this study underscores the significance of rigorous scientific research and collaboration among different sectors of society to address contemporary environmental challenges. Only through a comprehensive and coordinated approach can we protect ecosystems and ensure a sustainable future for future generations.

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Hosting institution

Politecnica Salesiana University

Ethics and security

The methodology outlined has been approved by the Bioethics Committee on Animals Experimentation of the Catholic University of Valencia San Vicente Mártir, ensuring that all procedures comply with the established regulations and ethical standards for the protection and welfare of the animals involved in the research. Suppl. material 2

Conflicts of interest

The authors have declared that no competing interests exist.

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Supplementary materials

Suppl. material 1: Sampling Design doi

Authors: Authors
Data type: Sampling scheme
Brief description: Sampling scheme with sampling dates and the number of bees collected per apiary and per hive.
Download file (71.82 kb)

Suppl. material 2: Bioethics committee Certificate doi

Authors: UNIVERSIDAD CATÓLICA DE VALENCIA SAN VICENTE MÁRTIR - ETHICS COMMITTEE ON ANIMAL EXPERIMENTATION
Data type: Certificate
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