Determination of microplastics in stingless bee, *Heterotrigona itama* (Hymenoptera: Meliponini)

by

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DECLARATION

I declare that this thesis entitled "Determination of microplastic in stingless bee *Heterotrigona itama* (Hymenoptera: Meliponini)" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature: ______________________________

Name : Muhammad Aiman Bin Bahari

Date : 6 JUNE 2024

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Determination of microplastic in stingless bee *Heterotrigona itama* (Hymenoptera: Meliponini)

ABSTRACT

Microplastics, resulting from the fragmentation or degradation of plastic materials, emerged as significant environmental pollutants with potential adverse effects on ecosystems and organisms. This research investigated the presence of microplastics within stingless bees (*Heterotrigona itama*), which are crucial for pollination and ecosystem maintenance. Despite their ecological importance, the impact of microplastics on these bees was not well understood. The objectives of this study were to assess the occurrence of microplastics in stingless bees and to characterize the types of microplastics present. To achieve these goals, a comprehensive approach was employed, integrating field sampling with laboratory analysis. Stingless bee colonies were collected from three local bee farms in glass container. Microplastic characterization was performed using Fourier-transform infrared spectroscopy (FTIR). This study provided valuable insights into the prevalence and types of microplastic contamination within stingless bees. There are 10 different kinds of microplastic was found resulting from the sample due to the environment status of the bee farm that can cause the effect of the stingless bee health and reproductive in the area.

Penentuan mikroplastik dalam lebah kelulut *Heterotrigona itama* (Hymenoptera: Meliponini)

ABSTRAK

Mikroplastik, hasil daripada pemecahan atau degradasi bahan plastic, muncul sebagai bahan pencemar alam sekitar yang ketara dengan potensi kesan buruk ke atas ekosistem dan organisma. Penyelidikan ini menyiasat kehadiran mikroplastik dalam lebah kelulut (Heterotrigona itama), yang penting untuk pendebungaan dan keseimbangan ekosistem. Walaupun ekologi mereka sangat penting, kesan mikroplastik pada lebah ini tidak difahami dengan baik. Objektif kajian ini adalah untuk menilai kejadian mikroplastik dalam lebah kelulut dan untuk mencirikan jenis mikroplastik yang ada. Untuk mencapai matlamat ini, pendekatan komprehensif telah digunakan, mengintegrasikan pensampelan lapangan dengan analisis makmal. Koloni lebah kelulut dikumpulkan dari tiga ladang tempatan dalam bekas kaca. Pencirian mikroplastik dilakukan menggunakan spektroskopi inframerah transformasi Fourier (FTIR). Kajian ini memberikan Pandangan yang berharga tentang kelaziman dan jenis pencemaran mikroplastik dalam lebah kelulut. Terdapat 10 jenis mikroplastik yang berbeza telah ditemui hasil daripada sampel kerana status persekitaran ladang lebah yang boleh menyebabkan kesan Kesihatan lebah kelulut dan reproduktif mereka yang berada di Kawasan tersebut.

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LIST OF ABBREVIATIONS

PVC	Polyvinyl chloride
FT-IR	Fourier-transform infrared Spectroscopy
MP	Microplastic
HCL	Hydrochloric acid
NaCL	Sodium chloride
PEN	Polyethylidenenorbornene

LIST OF SYMBOLS

MM	Millimetre
ML	Millilitre
%	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Microplastics are defined as plastic particles that have a length of less than five millimeters. The topic of environmental quality conservation and associated concerns has grown more concerning. Microplastics may be categorized as main and secondary microplastics based on their respective origins. Primary microplastics are intentionally produced with dimensions in the millimeter or sub-millimeter range. These particles are often found in many home items, including personal hygiene products like face cleansers and toothpaste. In the field of medicine, specifically in the context of drug vectors, it has been estimated that over 6% of the liquid skin-cleaning products marketed inside the European Union. Secondary microplastics are formed through the degradation of larger plastic particles when they are exposed to natural elements, undergo physical changes, and are subjected to biological and chemical processes. These processes can cause a decrease in the structural integrity of plastic debris, ultimately resulting in their fragmentation. However, this fragmentation may occur prior to the introduction of plastic into the environment.

The problem of plastic pollution has garnered significant attention because to its tangible manifestations, leading to the emergence of novel paradigms and comprehensive perspectives. Numerous individuals are intrigued by the realization that the presence of enormous yellow bees encompasses more than a singular species, which is often seen in flight. In Malaysia, there exist five distinct species that have been identified, namely *Geniotrigona thoracica, Heterotrigona itama, Lepidotrigona terminate*, *Tetragonula fuscobalteata*, and *Tetragonula leaviceps*. These species are frequently utilized in the meliponiculture industry within the country for the purposes of honey production and pollination (Kelly et al., 2014). The stingless bee has a resemblance to the honeybee in terms of its physical appearance and also exhibits similar social behavior by residing in colonies and possessing a distinct queen. The stingless bee is a significant ecological agent that plays a pivotal function in the ecological system. It serves as a prominent pollinator, facilitating the transfer of pollen between plants of different sexes, hence enabling fertilization, and aiding in plant reproduction.

Heterotrigona itama, popularly referred to as the dwarf stingless bee, is a species prevalent in many regions of Southeast Asia, including Malaysia. Stingless bees are well-documented for their foraging behavior, which involves collecting nectar from a diverse array of plant species. Recent research conducted by Wahyu et. Al (2023) has revealed the presence of microplastics in honey samples. However, the study did not elucidate the source of microplastic contamination. In response to this gap, the present study was designed to investigate whether stingless bees themselves were carriers of microplastics. This investigation aimed to determine if the microplastics detected in honey may be originating from the bees' own exposure to environmental contaminants, thus providing insights into the potential pathways of microplastic transfer within the ecosystem.

1.2 Problem Statement

The stingless bee plays a significant role in facilitating pollination processes for a wide range of crops, wildflowers, and trees. This crucial function supports the reproductive cycles of fruits and seeds, while also contributing to the maintenance of plant species diversity. Consequently, the stingless bee's activities foster the creation of diverse habitats for other species and contribute to the establishment of new ecosystems in certain locations. These bees are capable of producing honey, but in lesser numbers as compared to honeybees. The honey produced by stingless bees has a much higher therapeutic value owing to its distinctive chemical makeup. This honey has been historically used for its possible health benefits, making it a subject of interest in research and medical courses. Despite evidence of microplastic contamination in honey, there is insufficient information on whether stingless bees themselves are contaminated, which leaves a critical gap in understanding the sources and pathways of microplastic exposure in these pollinators.

1.3 Objectives

1. To determine the occurrence of microplastics within the stingless bee
2. To characterize the microplastic within stingless bee

1.5 Scope of Study

This study aimeds to gather samples of stingless bees from the 3 local bee farms from Kampung Kota, Kota Bharu, Kelantan (Suri Madu Kelulut), Kampung Paloh, Tendong, Pasir Mas, Kelantan (MR MADU KELULUT), No 23/A Jalan 5E/6 Sek 5E, Bandar Baru Bangi, Selangor (Madu Kelulut). In order to resume the study in a laboratory setting, it is necessary to retrieve the sample for the purpose of identifying the presence of microplastics within the stingless bees and determining the specific types of microplastics that the stingless bee ingests. The limitation of conducting research on the presence of microplastics in stingless bees solely within a laboratory setting, as opposed to a field sampling site, is primarily due to the requirement of specialized equipment such as a microscope, along with Fourier-transform infrared Spectroscopy (FT-IR) for accurate determination and observation of microplastic particles. The research endeavor included an observation of the surrounding ecosystem, in order to ascertain the probable presence of microplastics inside the habitat of stingless bees.

1.6 Significant of Study

The objective of the research efforts is to ascertain the presence of microplastic particles inside the stingless bee species. This study offers valuable insights into the phenomenon of microplastic pollution and its environmental dispersion. Specifically, it examines the role of stingless bees as bioindicators for detecting the presence of microplastics within various habitats. Conducting this study facilitates the comprehension of the possible impact associated with the absorption of microplastics on the well-being of stingless bees and the overall fitness of their colonies. The potential consequences include a decrease in food absorption by stingless bees, exposure to microplastics that are linked to chemical compounds, and the resulting physical harm. Given the crucial role that stingless bees play in pollination, it is essential to consider the potential effect of microplastic exposure on plant reproduction, crop production, and ecosystem health.

CHAPTER 2

LITERATURE REVIEW

2.1 Description of microplastic (MP)

Microplastics, which are microscopic plastic particles, are commonly found in several environmental mediums, including air and water. Microplastics are present due to natural factors such as solar radiation and the involvement of microorganisms in the breakdown process, which gradually leads to the degradation of plastic into minute particles known as microplastics. In their study, Thompson et al. (2004) provided a description of microplastics, which are little fragments of plastic, found within the sediments from the sea of European waters. According to Arthur et al. (2009), microplastics are defined as tiny plastic particles that have a size less than 5 millimeters (mm). The documentation of microplastic colors is seen as a crucial aspect of research due to the worry about organisms that exhibit a high likelihood of ingesting or consuming microplastics. This is attributed to their feeding habit, which often leads to confusion between microplastics and actual food resources (Wright et al., 2013).

Microplastics has been identified as a significant worldwide environmental issue (UNEP, 2009). The issue of plastic waste has emerged as a significant global concern due to its persistent nature and lack of inherent limitations (Shah et al., 2008). The majority of plastic materials possess the ability to transport certain types of toxins, which is contingent upon the specific sort of plastic being used (Mato, Y. et al, 2001). The degradation of microplastics typically occurs at a slower pace (Thompson et al., 2014). The phrase "transformation structure" is used to describe the chemical degradation processes including light, oxidation, and hydrolysis. On the other hand, biodegradation refers to the degradation process induced by living organisms, specifically bacteria.

2.2 Effect of MP to ecosystem & living biota

Microplastics are recognized as a significant source of pollution, posing a multifaceted issue with detrimental implications for both the environment & human health (Thompson et al., 2004). According to Jamieson et al. (2019), the pollution resulting from microplastics has a pervasive nature, capable of extending its reach to include many wilderness areas, even the deepest trenches of the ocean. In the context of terrestrial ecosystems, which include land-based communities including organisms and the interactions between biotic and abiotic factors, the issue of plastic and microplastic contamination has received very little attention. The sources of microplastics and plastic found on land involve the erosion of vehicle tires (Evangeliou et al., 2020), domestic and household activities, particularly when using cosmetic and cleaning products containing plastic substances (Murphy et al., 2016), synthetic fibers released during clothes and textile cleaning (Brownie et al., 2011; Napper and Thompson, 2016; Boucher and Friot, 2017), as well as coating and painting procedures (Takahashi et al., 2012). These activities are examples of human actions that contribute to the presence of plastic in terrestrial environments. The inadvertent combustion of plastic has the potential to result in the emission of plastic particles, often referred to as microplastics, into the atmosphere and its immediate vicinity. This occurrence may have detrimental consequences on neighboring water bodies, as shown by studies conducted by Gullet et al. in 2007 and Hale et al. in 2020.

The presence of microplastics in freshwater aquatic ecosystems is a multifaceted issue due to the diverse nature of these environments, encompassing rivers, ponds, and lakes. Each of these water bodies possesses distinct hydrological and chemical properties, yet all have the capacity to accumulate, submerge, and transport plastic and microplastic pollutants (Eerkes-Medrano et al., 2015). For instance, the flow of water may result in the improper handling of water and waste disposal, as plastic materials are introduced into freshwater environments via transportation mechanisms. This release of trash into the water ecosystem can subsequently lead to the deposition and runoff of plastic garbage (Xia et al., 2020). There is evidence from research indicating that storms are a contributing element to the contamination of aquatic ecosystems by microplastics. An increase in the quantity of microplastics within aquatic ecosystems has been seen during storm events, particularly during periods of rainfall, as opposed to pre-storm conditions. The research conducted by Xia et al. (2020) demonstrates that microplastic contamination is transported to the lake surface by rainfall. In addition to this, the deterioration of tires and potential runoff of road paint might contribute to the contamination of nearby aquatic ecosystems with microplastics (Horton et al., 2017).

The marine ecosystem is significantly impacted by microplastic pollution, which is primarily caused by plastic waste from industrial processes, and human actions in the coastal zone (such as fishing, aquaculture, marine industry, and tourism). This has been extensively documented in various studies, including the work of Lusher et al. (2017). The marine environment has distinct characteristics in terms of pressure, patterns, and oceanic water when compared to other aquatic ecosystems (Kane et al., 2020). Moreover, microplastics consist of a diverse range of polymers, each with its own distinct molecular structure. These microplastics also exhibit variations in terms of their size, shape, colors, and density, as discussed by Rochman et al. (2019). The impact of microplastic dispersion and sinking qualities on the transportation of such particles throughout marine ecosystems is a subject of interest. According to a study conducted by Koelmans et al. (2017), it was estimated through simulation modelling that approximately 99.8% of submerged plastic pollution has occurred since 1950. In the year 2016, a significant portion of this pollution, specifically 9.4 million metric tons of plastic particles, was found beneath the ocean surface layer. This movement of microplastics from the ocean surface layer to the deepest floor of the ocean's ecosystem was facilitated by transport mechanisms and hindered the natural habitat of various marine organisms (Porter et al. 2018). The issue of microplastic pollution in the ocean has become a significant cause for concern due to two primary factors. Firstly, the spreading of microplastics has the capability to absorb and subsequently release toxic substances, as evidenced by Gouin et al. (2011). Secondly, there is a high likelihood that living marine organisms, including seafood species, ingest these microplastics, as highlighted by Cole et al. (2011). This ingestion poses a potential risk to humans and other predators who consume contaminated seafood, as they may also be exposed to the toxic substances present in the microplastics, as discussed by Smith et al. (2018).

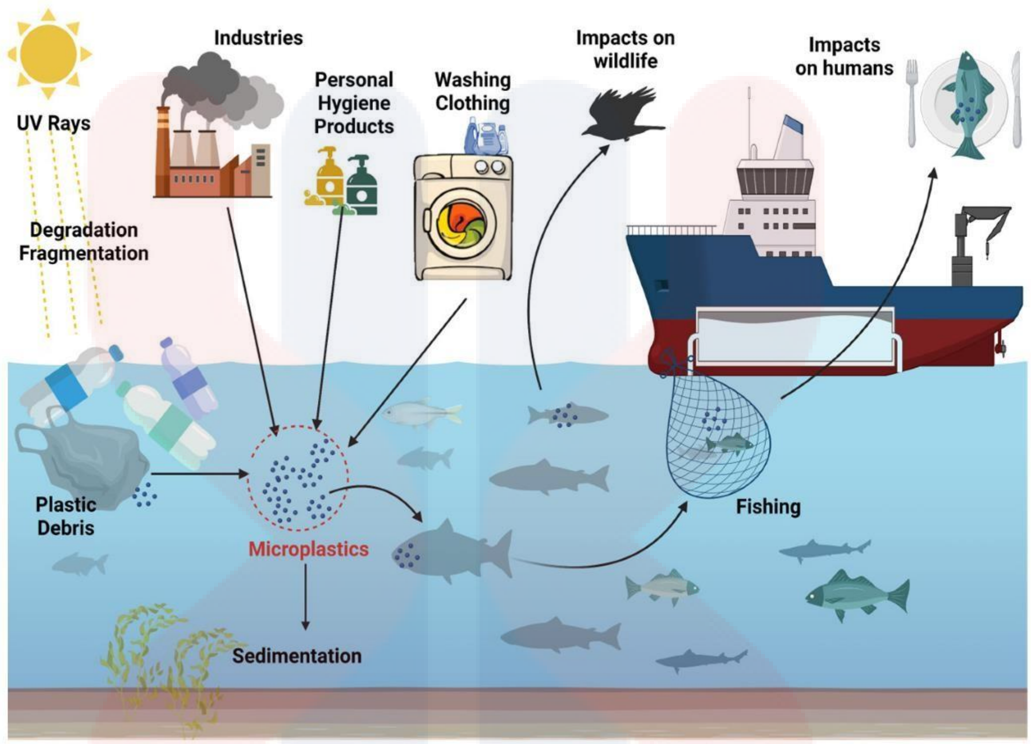


Figure 1: Cause and effect of microplastic pollution in marine ecosystem

In comparison to other environmental domains, the atmosphere remains relatively understudied in terms of quantifying microplastic contamination. The existing body of research on microplastics in the atmosphere suggests that the deposition of these particles in soil through wet processes strongly supports the theory that the atmosphere plays a significant role as a pathway for the global dispersion of microplastic pollution. This is achieved through the transportation of microplastics from urban areas to more remote areas (Dris et al., 2016; Allen et al., 2019). For instance, recent studies have shown the presence of microplastics in distant regions such as the Arctic (Bergmann et al., 2019) and the outermost layers of the ocean (Wang et al., 2020). Despite the wide variety of shapes, sizes, and molecular structures of microplastic particles, their typically low material density, tiny shape, and high surface area facilitate their easy entry into the atmosphere and subsequent suspension or adaptation (Dris et al., 2016; Wang et al., 2020). According to Bank and Hansson (2019), the transportation mechanisms of microplastic pollution in the atmosphere are similar to those of carbon, nitrogen, and mercury. This finding suggests that plastic particles follow comparable paths within the overall environmental system. Living organisms, including humans, are susceptible to exposure to microplastics via several pathways such as water and air, as well as through the consumption of food products that contain these particles (Cole et al., 2011). One of the key concerns pertains to the presence of toxic substances within microplastics, which have the potential to contaminate the environment and adversely affect organisms that come into contact with them. The ubiquity of microplastics as a pollutant has resulted in significant exposure to humans and other living organisms through both ingestion and inhalation, thereby posing potential health risks (Prata et al., 2020).

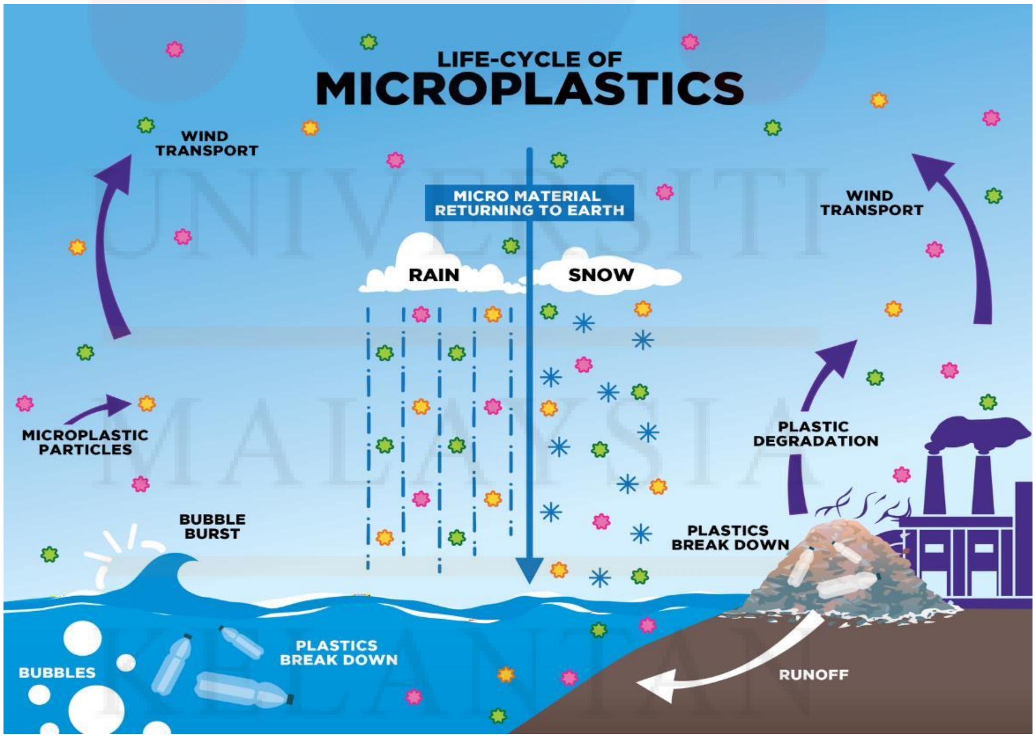


Figure 1.1: Global threat of microplastic cycle pollution in atmosphere

2.3 Previous studies MP and insects

Table 1: Previous studies related on microplastic and insects

Year	Author	Previous studies
2023	Jie Shen, Boying Liang, HuiJin	The impact of microplastics on insect physiology and indication of hormesis
2021	Gabriele Rondoni, ElenaChierici, Alberto Agnelli,Eric Conti	Microplastics alter behavioural responses of an insect herbivore to a plant-soil system
2021	Kai Wang, Jihuan Li, LiuweiZhao, Xiyan Mu, ChenWang, Xiaofeng Xue,Suzhen Qi, Liming Wu	Gut microbiota protects honey bees (*Apis mellifera L.*) against polystyrene microplastics exposure risks
2023	Juan-Ying Li, Yang Yu, Nicholas J. Craig, WenhuiHe, Lei Su	Interactions between microplastics and insects in terrestrial ecosystems – A systematic review and meta-analysis
2021	Yanchun Deng, Xuejian Jiang, Hongxia Zhao, SaYang, Jing Gao, YanyanWu, Qingyun Diao, and Chunsheng Hou	Microplastic polystyrene ingestion promotes the susceptibility of honeybeeto viral infection
2022	Marshall W. Ritchie, Alexandra Cheslock, Madelaine P.T. Bourdages, Bonnie M, Hamilton, Jennifer F. Provencher, Jane E. Allison, Heath A. Macmillan	Characterizing microplastic ingestion, transformation, and excretion in insects using fluorescent plastics

2.4 Description of stingless bee

According to Siregar et al. (2016), stingless bees are considered to be very efficient pollinators in comparison to other insect species. There are several kinds of bees worldwide, including the Meliponines, which are often referred to as stingless bees. The Meliponines represent the largest assemblage of eusocial bees, with an extensive variety of over 600 recognized species (Lavinas et al., 2019). The stingless bee and honey bee have similarities, but they vary in terms of their morphology and size. Despite these differences, both species perform a crucial role in facilitating plant dispersal via pollen fertilization, so ensuring the continuing survival of plant species. The stingless bee species exists in large colonies consisting of many workers, males, and a solitary queen that governs them (Jailani et al., 2019). Typically, the queen is larger in size compared to the males and worker bees, and due to their inability to fly, they rely on the protection provided inside the colony (Efin et al., 2019). According to Miller et al. (2009), female bee workers primarily engage in foraging for food sources, such as nectar, whereas male bees serve as mating partners for the queen.

Queen weigh more than workers 2 to 6 times and they have long size of body (Gruter et al. 2017). Compared to workers, queen have small head, eyes, and shorter wings but have wide size of thorax and longer antennae (Schwarz. 1984). Most individuals in a stingless bee colonies are responsible to perform more tasks to maintain the colony in a good state, most important things they need to do is building nest structures. Stingless bee nest is pre-existing cavities such as holes in the ground and sometimes in the ground besides termite's nests (O'Toole, C. 2013). For the material that they're use for making the nest by using a secrete wax that mix with resin and gum that are collected by workers bee from plant wounds matter and mixed it with animal faeces or mud for cell construction of their nest (O'Toole, C. 2013).

According to O'Toole (2013), sting-less bees have reduced functionality due to their sting compared to other bee species. However, despite this limitation, they are still able to effectively defend their colonies. The colonies possess huge stores of nectar and larvae, enabling them to overwhelm intruders such as beetles attempting to infiltrate their hive. In defense, they use a biting strategy characterized by aggressiveness. The mandible gland of stingless bees has an additional purpose, wherein it aids in the communication of food sources throughout the colony. When these bees return from environments rich in pollen or nectar, they leave scented trails by depositing glandular secretions. These trails effectively direct other members of the colony, facilitating the tracking of food supplies.

2.4.1 Morphology of the stingless bee

The stingless bee is classified as a eusocial insect, which is characterized by its communal living arrangements inside a hive (Michener, 2017). Stingless bees are recognized for their major contribution to crop pollination (Kelly et al., 2014). Additionally, these bees are known to generate substantial quantities of nectar and resin (Francoy, 2009; Mohamad et al., 2020). A study was conducted at the Entomology Laboratory of Gadjah Mada University (Smith, 2012) to investigate the morphology of stingless bees. Various species of stingless bees were selected as specimens for morphological observations, focusing on the coloration of different body parts such as the head, thorax, wings, and legs. Detailed descriptions of each species were provided along with accompanying images of the respective body parts.

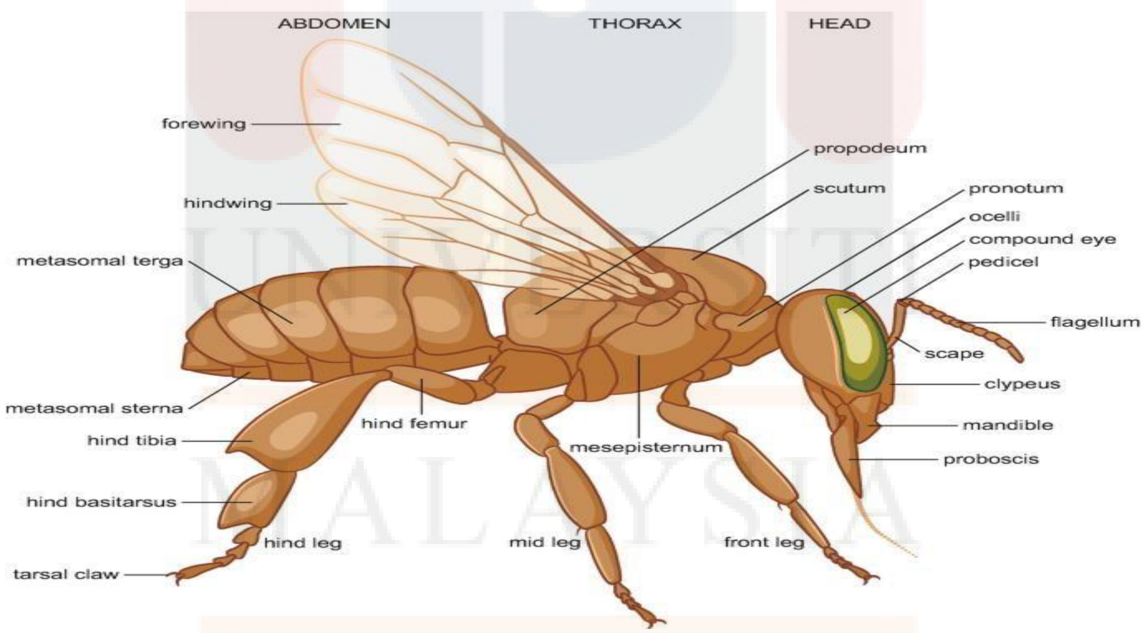


Figure 2: Morphology of stingless bee

The study focuses on the anatomy of the selected stingless bee species, namely *Heterotrigona itama*. The diameter of the bee's head is almost twice the length, and it is coated with setae. The stingless bee has two compound eyes that serve as a means of seeing, enabling it to get information on its surrounding environmental conditions. Additionally, the head of the bee exhibits a blackish hue, while the ocelli, which are simple eyes, also share a similar blackish color (Cockerell, 1918). The organisms possess antennae that have a morphology resembling small rod-shaped structures, which serve as sensory organs for olfaction, gustation, and a distinctive auditory capability. The stingless bee has a mandible with a single tooth, which serves the purpose of chewing wood, particularly when they need to modify their hive or when they use it to eat pollen in order to produce wax for constructing their combs (Cockerell, 1918).

2.4.2 Roles and feeding guilds of the stingless bee

According to Ollerton (2011), bees fulfill a crucial function within ecosystems as pollinators. The author asserts that around 90% of all angiosperm flower plants rely on bees and other animals for pollination. The stingless bee serves as a consistent pollinator, exhibiting a tendency to frequent diverse environments including a wide range of flowering plant species. For instance, according to Ramalho's (2004) findings, stingless bees contribute to 70% of the pollination activity in environments characterized by a diverse range of blooming plants, despite the fact that they represent only 7% of the total species diversity. The majority of stingless bees engage in the collection of pollen and nectar from a diverse range of plant species, hence contributing to the preservation of genetic diversity among flowers within their habitat (Slaa et al., 2006). Some species of stingless bees exhibit predatory behavior by invading the nests of other colonies to gather resources such as honey, wax, resins, and even larvae, which serve as a source of carbohydrates for their nutritional requirements (Nagamitsu et al., 1997).

Figure 2.1: Stingless bee carrying crop/pollen and deposited it for newborn flowers plant

2.4.3 Type of method apply in research studies

The research methodology used in this study mostly excludes the use of plastic materials to ensure that the gathered samples of stingless bees remain uncontaminated and unexposed to plastic and microplastic substances. In order to get the necessary samples, it is essential to conduct a systematic collecting process. The stingless bee specimens should be immediately gathered from their respective nests or hives, and thereafter stored in vials or PVC tubes for preservation. The process of chemical digestion was used in order to extract microplastic particles that had been ingested by stingless bees in the collected samples. Which include hydrochloric acid 30% (HCL), were utilized to dissolve the organic components and separate the plastic particles present in the samples (Catarino et al., 2020). To ascertain the specific microplastic or plastic polymer present in stingless bees, it is necessary to employ Fourier-transform infrared (FTIR) spectroscopy. This analytical technique enables measurement and comprehension of the composition, thereby facilitating the development of strategies to mitigate the contamination of these pollinators habitats by observe plastic polymers (Wang et al., 2017).

CHAPTER 3

MATERIALS AND METHODS

3.1 SAMPLING LOCATION

Table 2: Sampling site of stingless bee

Sample 1 (Suri Madu Kelulut)	Sample 2 (MR MADU KELULUT)	Sample 3 (Madu Kelulut)
		
Kampung Kota, Kota Bharu, Kelantan	Kampung Paloh, Tendong,Pasir Mas, Kelantan	No 23/A Jalan 5E/6 Sek 5E, Bandar Baru Bangi, Selangor

The stingless bees (*Heterotrigona itama*) sampling was conducted in 3 different bee farms. Sampling for sample 1 (Suri Madu Kelulut) was conducted in Kampung Kota situated in the Kota Bharu district. The geographical coordinates of Kampung Kota are 6°04'40.7"N latitude and 102°13'46.2"E longitude. This location was chosen for its suitability for stingless bee sampling due to nests that have been build up by beekeepers themselves in a rural area, the village was characterized with diverse of organic matter in the environments that was good for provided the results of the findings needed. Sampling for sample 2 (MR MADU KELULUT) was conducted in Kampung Paloh, Tendong situated in Pasir Mas district. The geographical coordinates of Kampung Paloh are 6° 04' 11.8''N latitude and 102°12' 38.9''E longitude. This location was chosen because it has features with various kind of plantation within the environment which offer the natural habitat and food resources like pollen and nectar for stingless bees. Sampling for sample 3 (Madu Kelulut) was conducted in No 23/A Jalan 5E/6 Sek 5E situated in Bandar Baru Bangi district. The geographical coordinates of the sampling location are 2°57'19.9''N latitude and 101°47'09.9''E longitude. This location was chosen for its bee farms in a urban areas to differentiate findings of the what kinds of microplastic that might be occurred between rural and urban areas. All the samples that are contain will be keep in a glass container to avoid the sample from being contaminate by any other microplastic sources from the surrounding environment.

Figure 2.2: Stingless bees sample contain in glass container

3.1.1 MATERIALS

In this research studies, researcher will be focusing more to do a lab work after have collected the sample from 3 local bee farm. This is the following equipment that have been listed will be use when conduct the research studies:

Table 3: Shows materials and apparatus use in lab work conduct

Laboratory materials & apparatus	1) Chemical substance: • Sodium chloride (NaCL) • Hydrochloric acid 30% (HCL) • Distilled water 2) Lab materials: • Tube PVC • Glass beaker • Glass petri dishes • Conical flasks • Glass fiber filter paper • Vacuum filtration • Digestion tubes • Separation funnels 3) Lab Instruments: • Vacuum pump

	• Laminar flow cabinet • Stereomicroscope • Fourier-transform infrared spectrometer (FTIR) • Microplate Shaker • Filtering flask • Buncher funnel • Silicon tubing hose • Analytical balance 4) Others equipment: • Lab coats (others type thanfiber coats) • Gloves (others than macrofibre's glove) • Safety goggles • Forceps • Microscope slides • Measuring cylinder • Mask 5) Software • OMNIC Thermo scientific

Figure 3: Shows Fourier-transform infrared spectrometer (FTIR) apparatus to identify polymer type consist in sample

3.2 METHODS

3.2.1 Stingless bee sampling

In order to ensure the integrity of collected specimens, researchers must take precautions to avoid any potential contamination from plastic polymers found in the surrounding environment. One way to achieve this is by using glass beakers or containers to house the specimens and opting for aluminum as sealing materials instead of plastic seals. To obtain the sample, stainless steel forceps are utilized instead of plastic tweezers for handling the bees and transferring them into a container. This approach reduces the potential risk of plastic particle exposure. In addition, cotton gloves are used during the sample collection process instead of synthetic fabric gloves. The stingless bees collected from various local bee farms have specific weights, typically around 5 grams for each replicate for each local bee farm, which are then placed in glass containers or PVC tubes. The amount of stingless bee samples collected follows a suggested ratio of 3:3:3 for each local bee farm. It is not necessary for the samples to be fresh and alive; even dead samples can be collected.

Figure 3.1: 5 grams stingless bee sample contain in glass container

Figure 3.2: Cover and label the Petri dish using aluminium foil that are used to store the stingless bee sample

3.2.2 Isolate microplastic & data analysis

Before the digestion of the stingless bee sample are done, we need to isolate the microplastic occurred on stingless bee body or morphology to avoid a bias result contain from outer side of microplastic found and within the stingless bee one. To isolate the microplastic from outer layer of stingless bee sample, we need to use a microscope to see the microplastic with clear vision since the plastic particle was too small, after the microplastic have been found, we can isolate the microplastic using the stainless-steel forceps and then wash the sample using the distilled water to clearing the microplastic from the sample body. When trying to extract microplastic particles from the sample, a specific chemical substance is used based on the quantity or ratio of the sample taken. For instance, Hydrochloric acid 30% (HCL) is utilized to dissolve the sample and separate the plastic particles. The use of sodium chloride (NaCL) helps in the separation of microplastic particles by causing them to float in separation funnels, these 2 chemicals are combined to digest the sample on each replicate sample with same amount which are consist 60ml use of hydrochloric acid and 20ml of sodium chloride that have been dilute with distilled water.

These particles are then mixed in a microplate shaker for a period of 120 hours or 5 days long to ensure thorough mixing of the small volume samples and covering the sample that have been digest in the conical flask using the aluminum foil to avoid the sample from contaminated from the plastic particle from surrounding environment. This preparation allows for the collection and further analysis of the microplastic particles. To get a fresh sample after the digestion process have been done, we're using the vacuum pump to filter the sample that have diluted with chemical substance added through filtration with filter paper that are specialized use to gain microplastic sample (non-sterile cellulose acetate membrane type of filter paper by Bioflow Lifescience Sdn.Bhd) to put under FTIR to determine the presence of microplastic substances, we will employ a stainless-steel forceps needle. By heating the forceps and carefully touching the substance, we can identify whether it contains microplastics. If the substance melts, it indicates the presence of microplastic particles within the stingless bee. To characterize the type of microplastic and its presence in the sample, we will use a stereomicroscope to examine whether there are any actual microplastic pieces present after isolating them from the stingless bee. Later, it underwent Fourier-transform infrared spectroscopy (FTIR) analysis and well-prepared the occurrence of the microplastic inside the stingless bee sample from these 9 replicates using OMNIC software to gain the result on a presence of the microplastic and prepared the display result using the bar chart and pie chart. This analysis aims to identify the specific type of microplastic ingested by the stingless bee sample.

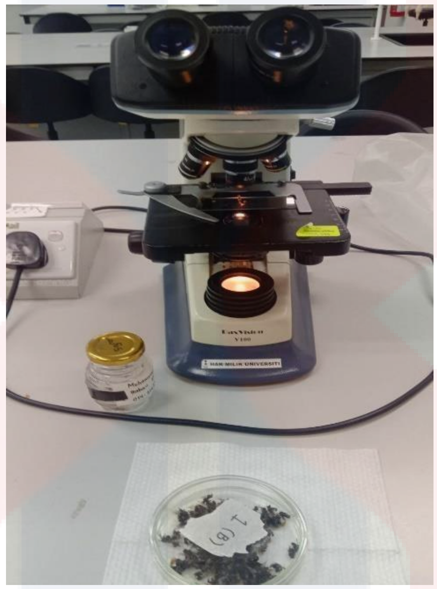


Figure 3.3: Observe and isolate the microplastic presence on outer layer body of stingless bee sample using forceps

Figure 3.4: Samples that have been digest were put in the shaker for 5 days/120 hours with revolutions per minute (RPM SV 100) and temperature SV was in 26.9 °C

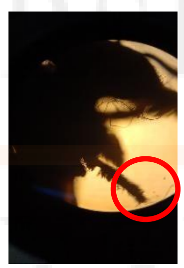
CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Type of microplastic on stingless bee (*Heterotrigona itama*)

The morphology of stingless bee from 3 local stingless-bee farms were inspected under the microscope. Using the stereomicroscope, the samples were examined meticulously, focusing on their morphology, including the head, thorax, and abdomen, to isolate microplastics prior to the digestion process. This approach was employed to prevent potential biases arising from the outer layers of the stingless bee bodies.

Table 4: The recorded microplastic found under the microscope from 3 local bee farmswith all sample replicate

Microplastics		
Replicate 1 Sample	Replicate 2 Sample	Replicate 3 Sample
 Fibers  Fibers	 Fibers  Fibers	 Fibers  Fibers

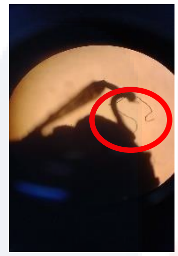
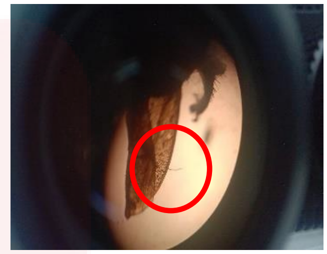
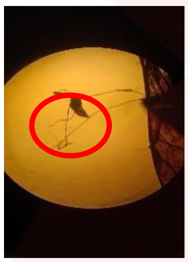
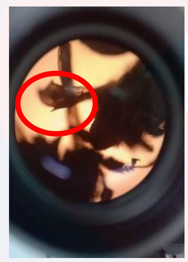
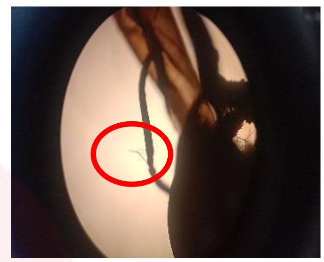
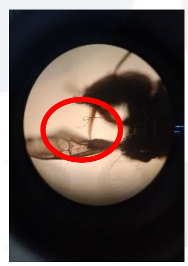
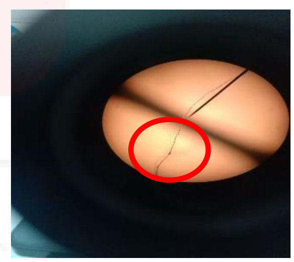
 Fibers  Fibers	 Films  Fibers	 Fibers
 Fibers  Fibers	 Fibers  Fibers	 Fibers
 Fibers  Fibers	 Fibers  Fibers	 Fibers

Table 5: Polymer types and colour found using manual counts of physical detection of microplastic on Stingless bee sample morphology

Polymer type	Colour	Total
Fibers/Filaments	White/Transparent	147
Films/Sheets	Light red	1

4.2 Microplastic presence in stingless bee (*Heterotrigona itama*)

Based on the result, most of the sample replicate of stingless bee has microplastic occurred on the outer layer of stingless bee morphology except for the replicate on sample 3(c) there are none microplastic found in this sample replicate. Microplastics were found attached to the head, antenna, legs, including fore and hind wings. The common type of microplastic that can be verified on stingless bee body was Fibers or Filaments and one of the other kinds which is Films or Sheets that only have been found in replicate 2(b). Fibers and Filaments are a thin and look-alike elongated strand of microplastics that can be cause by synthetic textiles, same with Films or Sheets found that have a red colour also thin and flexible microplastic particles that can originate form from the degradation of plastic bags, wraps, and other packaging materials.

The probability of causing this microplastic to attached on the structure of the stingless bee is likely due to the microplastic pollution factor that occurs in the bee farm environment, even if the bee farm is located in an urban or rural area. microplastics can still contaminate stingless bees through environmental condition such as air, water, and soil or even humans themselves causing exposure through the process of how they want to extract honey from stingless bee nests or activities in the local area that cause degradation to occur such as waste burning waste activities. The findings of this study provide compelling evidence of the microplastics presence within the stingless bee colonies in 3 different bee farms.

Although only two types of microplastics were observed on the body of stingless bees, the potential existence of additional microplastic types within the bees could not be ruled out. This possibility arises from potential exposure pathways such as ingestion, where microplastics can impact bee health. For instance, elongated fibers may cause physical blockages in the digestive tract, leading to reduced nutrient absorption and internal injuries, while films may facilitate the leaching of chemicals. Microplastics have the propensity to absorb chemicals from their environment or other sources of toxicity, which can lead to adverse effects upon ingestion. Such effects could include behavioral changes, alterations in foraging patterns, and impacts on reproductive success.

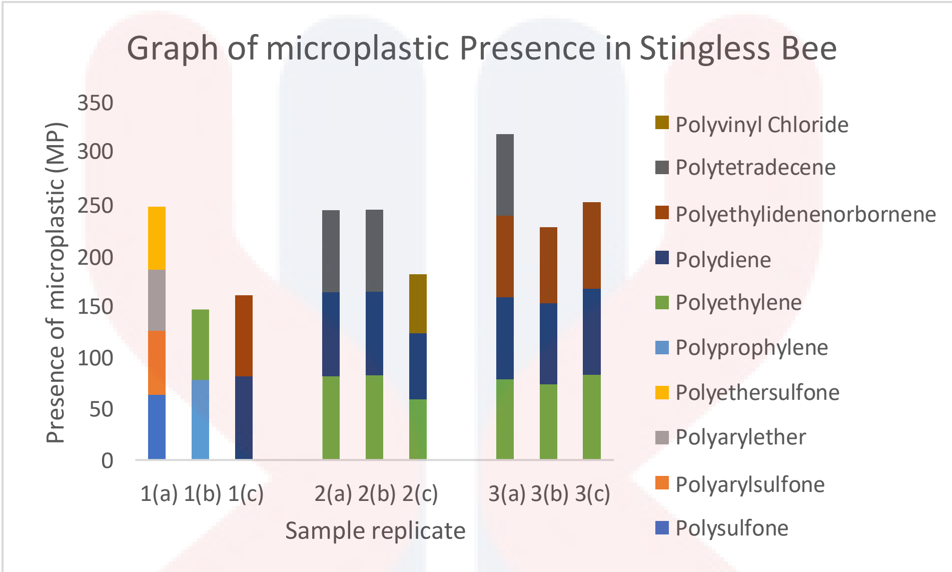


Figure 3.5: Type of microplastic recorded in stingless bee sample

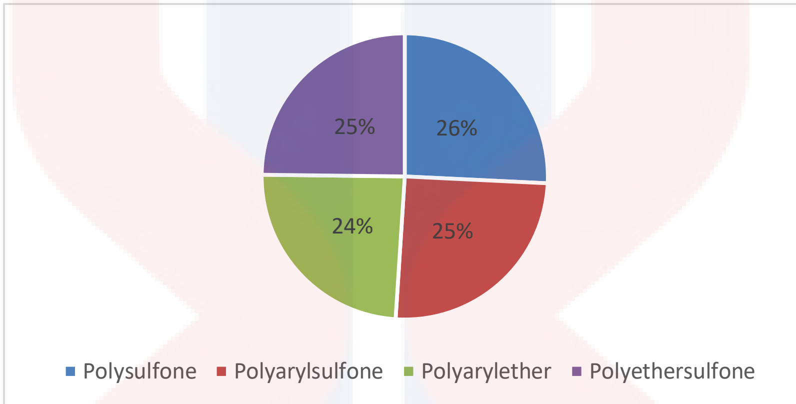
Based on the figure 4.4, indicates there was 10 different microplastic types that have been found among of these 9-replicate sample from each 3 stingless bee local farm, sample 3(a) have the highest rate presence of microplastic within the stingless bee with 4 kind of types microplastic presence which are Polyethylene, Polydiene, Polyethylidenenorbornene, and Polytetradecene with total 317.9 microplastic in the sample. While the smallest amount of microplastic presence within the stingless bee in other replicate is coming from sample 1(b) with only 2 kinds of microplastics presence which are Polypropylene and Polyethylene with total number of microplastic presence is 146.64 microplastic in the sample.

Table 6: Polymer types found resulting from FTIR

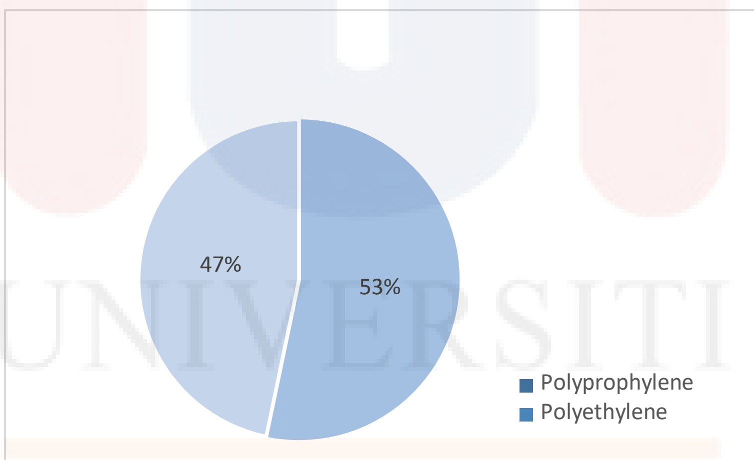
Types	Suri Madu Kelulut (Sample 1)	MR MADU KELULUT (Sample 2)	Madu Kelulut (Sample 3)
Polysulfone	145	0	0
Polyarylsulfone	25	0	0
Polyarylether	60	0	0
Polyethersulfone	55	0	0
Polyprophylene	70	0	0
Polyethylene	80	205	235
Polydiene	0	210	230
Polyethylidenenorbornene (PEN)	75	0	270
Polytetradecene	0	195	75
Polyvinyl Chloride	0	60	0
Total	560	670	810

Table 7: Shows pie chart result present microplastic presence in 9 replicate stingless bee sample

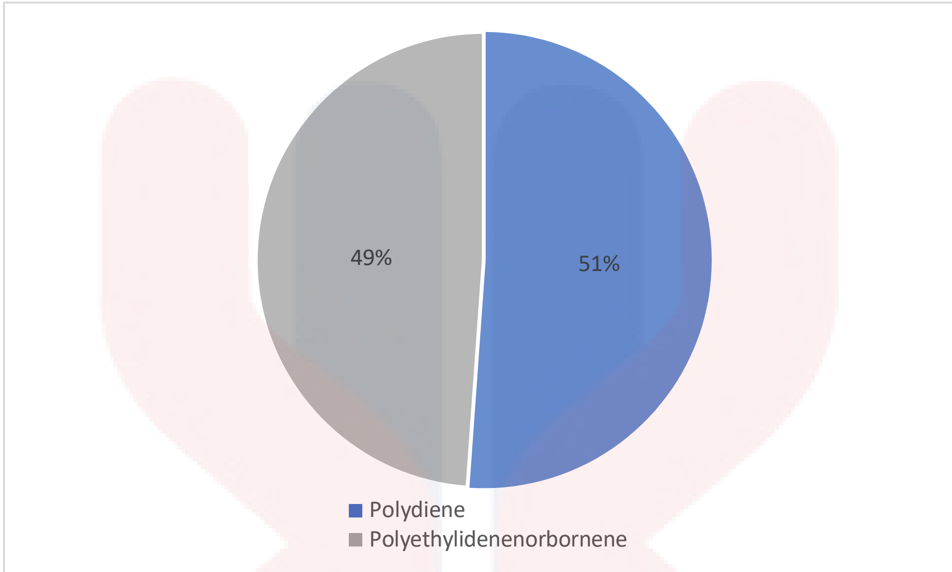
Sample replicate



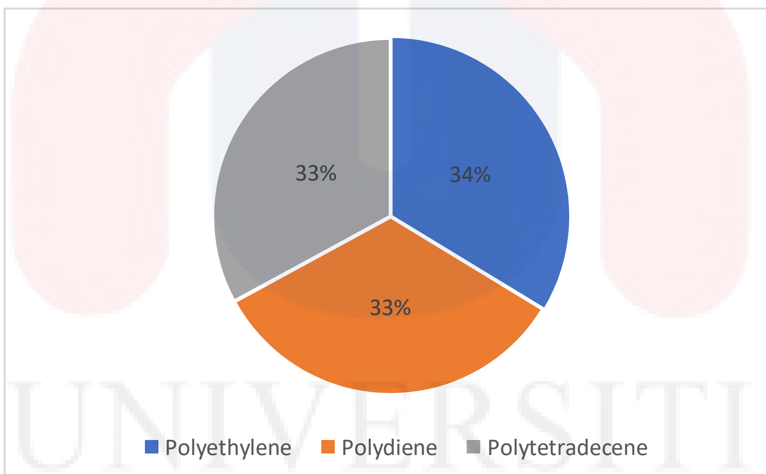
Sample 1(a)



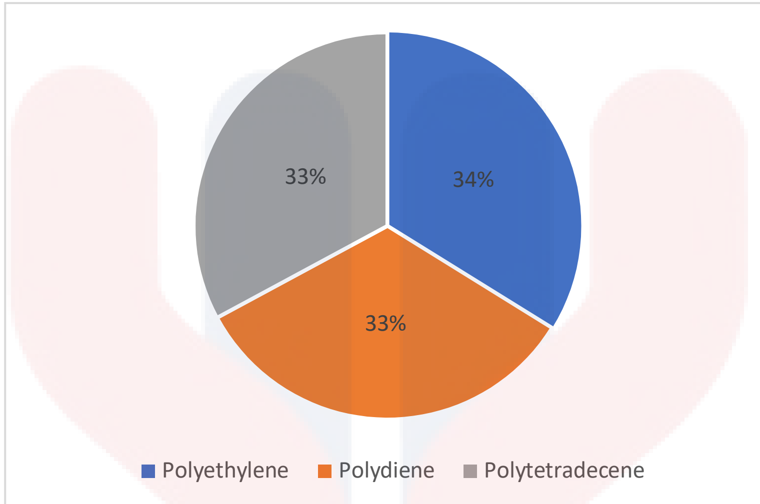
Sample 1(b)



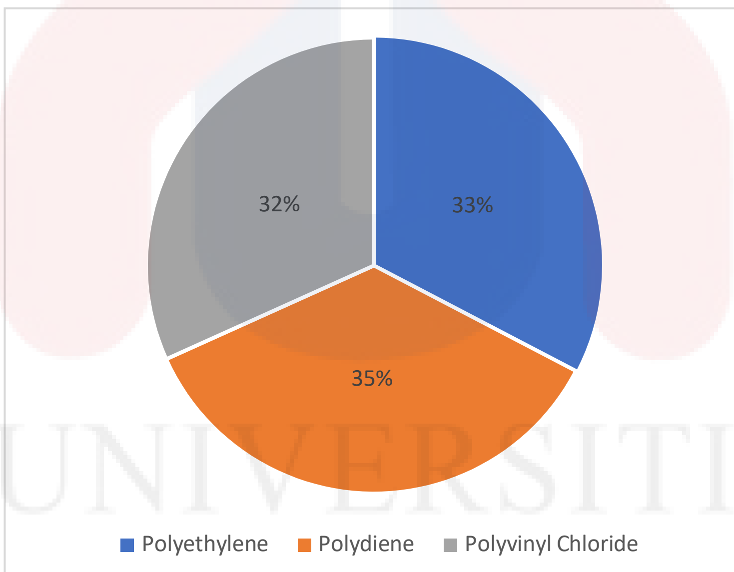
Sample 1(c)



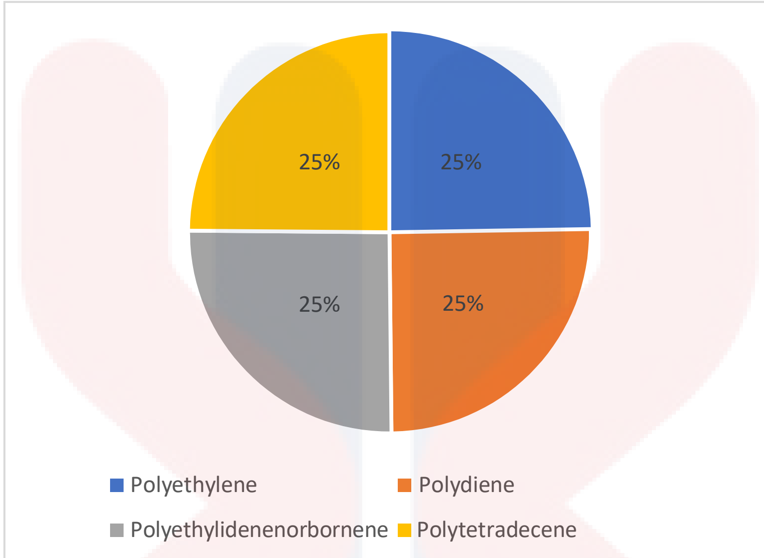
Sample 2(a)



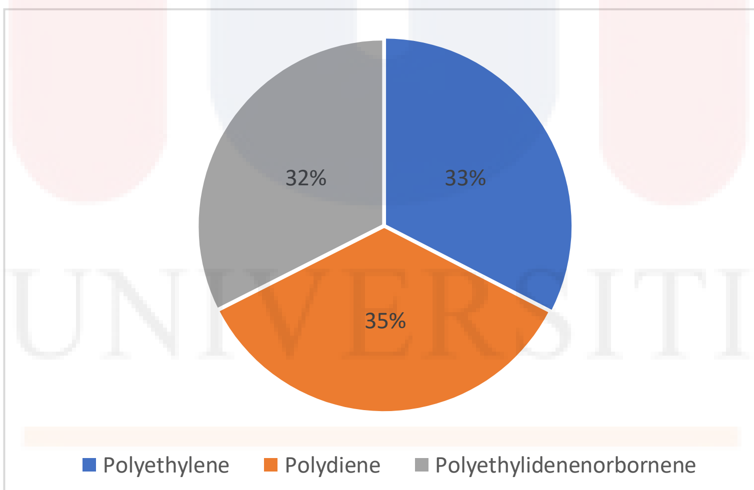
Sample 2(b)



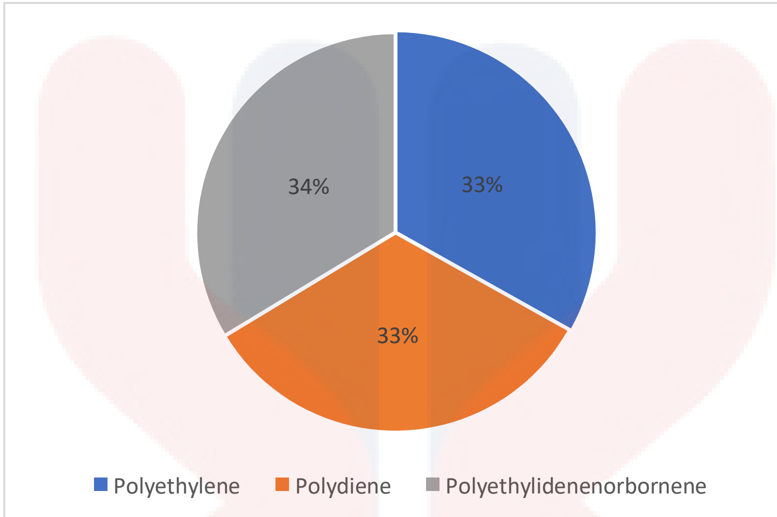
Sample 2(c)



Sample 3(a)



Sample 3(b)



Sample 3(c)

The pie chart that shows the result from sample 1(a) is displaying the relative abundance of four types of engineering microplastic polymer or man-made polymer that found in the stingless bee sample. These polymers are polysulfone, polyarylsulfone, polyarylether, and polyether sulfone. The highest rate of microplastic that contaminate in stingless bee sample 1(a) is polysulfone (63.57, 26%) with the others kind that are just always almost the same percentage of presence in the sample. Polysulfone (PSF) was widely used in the manufacture of electrical components and automobile components (Fu et al., 2018) These high-performance polymers are widely used in various industrial and consumer application due to their exceptional thermal, chemical, and mechanical properties. The presence of these microplastics in stingless bees is concerning as they are synthetic materials that do not occur naturally in their environment (Lara et al., 2020). Stingless bee could inadvertently ingest or come into contact with these microplastics through various pathways, such as contaminated nectar, pollen, or water sources. Factors like proximity to industrial or urban areas, improper waste management practices, and environmental degradation of plastic products could contribute to the contamination of the stingless bee habitat with these microplastics. The ingestion or exposure to these microplastics can potentially affect the health and behavior of stingless bee. Some potential impacts include physical obstruction or abrasion of their digestive system, disruption of their feeding and foraging behavior, and potential toxic effects from leaching of the chemical additives or monomers present in these polymer (Guzzetti et al., 2020).

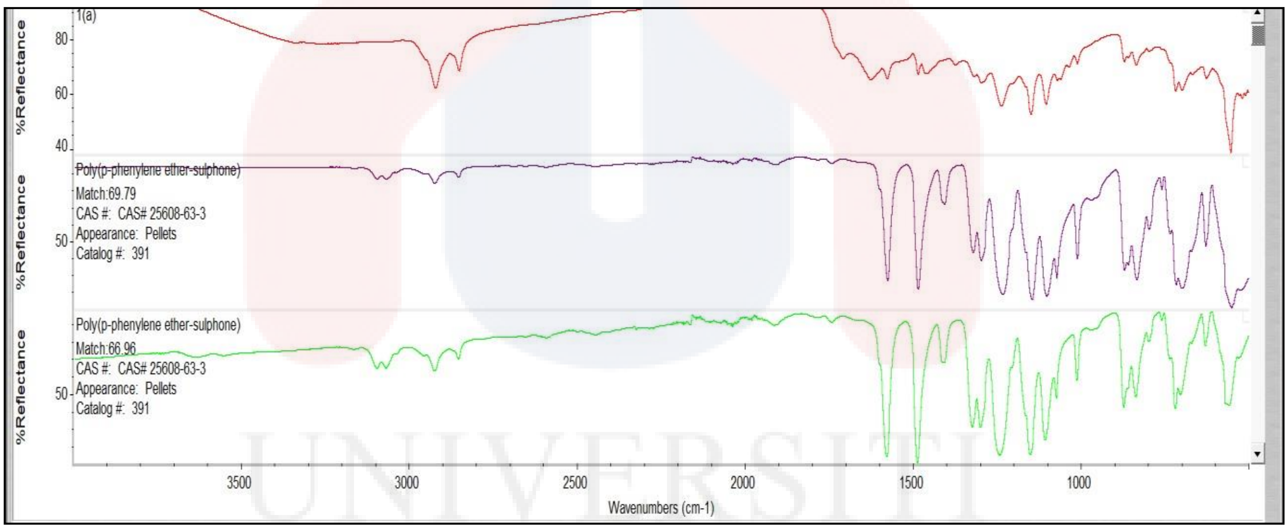


Figure 3.6: Results shows Polyprophylene, Polyether, Polysulphone and Polyphenylene composition in stingless bee (replicate 1) from Suri Madu Kelulut using FTIR

Resulting from sample 1(b), there is only 2 common plastic polymers are found which are polypropylene and polyethylene. These polymers are widely used in various consumer and industrial products due to their low cost, versatility, and durability (Oliveira et al., 2020). Polypropylene is primarily used in packaging materials, automotive components, household items, and textile fibers. Polyethylene, on the other hand is commonly found in plastic bags, bottles, containers, and construction materials (Nazir et al., 2021). The presence of these microplastics in stingless bee habitats raises concerns about potential contamination and adverse effects on these important pollinators. Presence of these microplastic because of improper waste management and degradation or bio-degradation of the plastic products in their environment. Same with the previous discussion stingless bee may inadvertently ingest the microplastic due their surrounding environmental that have been pollute with these kind of microplastic, since this sample 1 replicate was collected in a rural areas, that may be the cause why there are a lot of abundance for polypropylene (53%) compared to polyethylene (47%) due to most of the household items are use in the area, textile fibers and include packaging materials use to commercialize the honey products from the bee farm (Gorowara, M et al., 2022). Stingless bee may affect and cause them the negative impact like can cause harm for their digestive systems and disrupt their feeding guild and foraging behavior from the misjudge of ingestion microplastic with food and they could be exposed by toxic that are contaminate in the microplastic

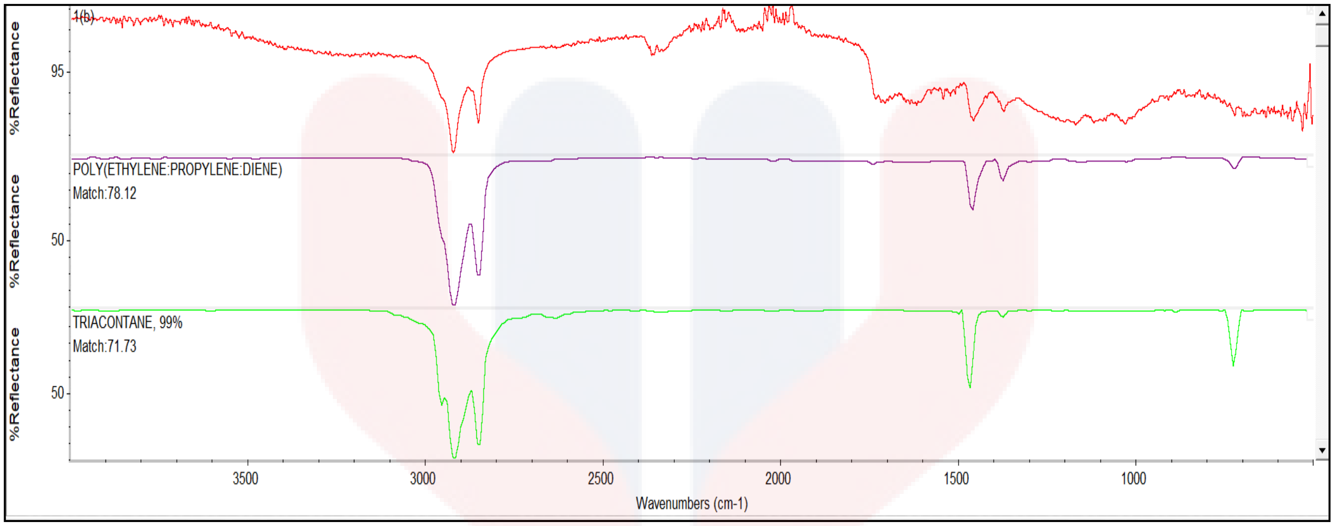


Figure 3.7: Results shows Polyethylene, Polyprophylene, Polydiene and Triacontane 99% composition in stingless bee (replicate 2) from Suri Madu Kelulut using FTIR

Sample 1(c) also only can find two types of microplastic which are polydiene and polyethylidenenorbornene (PEN) within the stingless bee. Polydiene is primarily used in the tires manufacturing because polydiene rubbers proved an excellent resistance to abrasion, heat and weathering, examples of the products that manufacture using polydiene such as car tires, truck tires, aircraft tires, and other rubber products that require high resilience and durability (Yeung, C. W. S. et al., 2021). Polydiene exposure rate in stingless bee for this sample replicate is 51% rather that are more a lot than PEN (49%) due to probably the rural areas of local bee farm near to the roadside where the vehicle are mostly used that cause the creation of microplastics from tire that the vehicle use and abrasion that releasing significant amount of microplastic particles onto roads nearby surroundings area and the other reason is may be lead to improper disposal of used tires and products in the area got degradation (Lim, J. Y. C. et al., 2021).

Polyethylidenenorbornene (PEN) is a plastic that are used in the production of high-performance electronic components due to its excellent thermal stability and electrical insulation properties, examples of these manufacture are printed circuit boards, semiconductor packaging material and electrical wires and cables (Urbanek et al., 2022). Polyethylidenenorbornene (PEN) also used for manufacture various filter application such as water treatment and industrial filtration process.

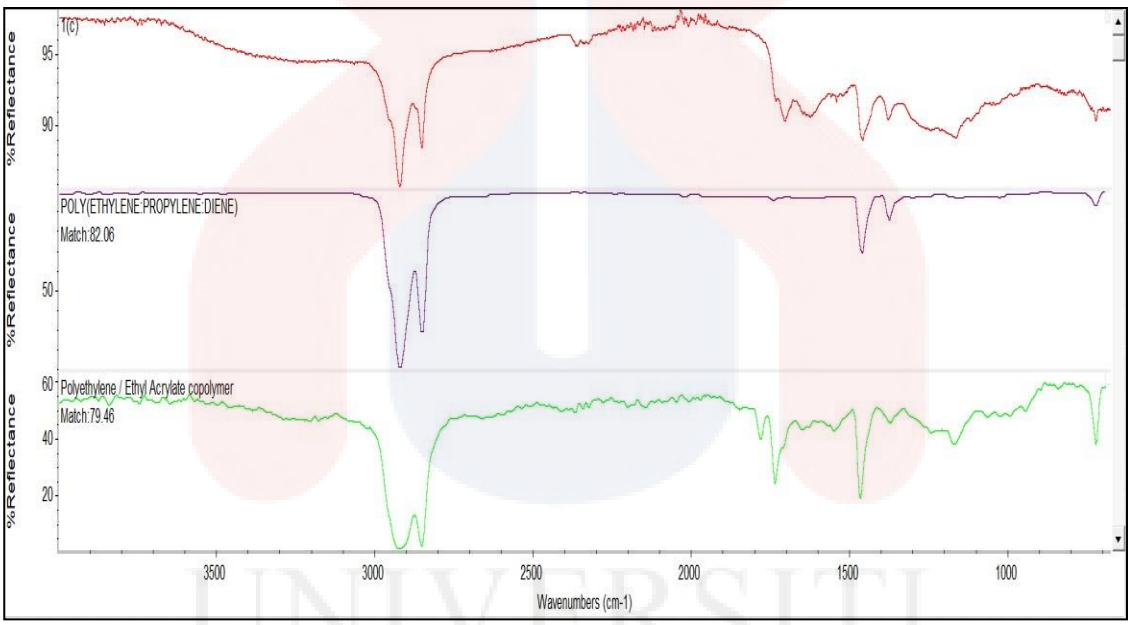


Figure 3.8: Results shows Polyethylene, Polyprophylene, Polydiene and Polyethylene/Ethyl Acrylate copolymer composition in stingless bee (replicate 3) from Suri Madu Kelulut using FTIR

Sample 2 is another stingless bee sample that have been collected in another rural areas of local bee farm with 3 replicates. Sample 2(a) and sample 2(b) both shows 3 kinds of same microplastic presence with same tabulate abundance which are polyethylene, polydiene, and polytetradecene. Polyethylene occurrence in a 2(a) sample is 82.02, 34% which means highest than other 2 microplastic found in the sample that only stand with occurrence amount 81.29, 33% (polydiene), and 80.13, 33% (polytetradecene). Polyethylene is the most kind of microplastic found in the sample because the bee farm was manufacture near the house of the owner, since polyethylene was come from plastic bags, food or drinks, containers, and any other medical application contain that have been degradation nearby (Gallo, C.A. et al., 2021). High presence of this polyethylene is because of the thermal stability of the plastics that are easily can be melted in a high temperature that due to the degradation or biodegradable from the environment status that can easily pollute the ecosystem surrounding of the bee local farm and contaminate the stingless bee including their honey for commercialize. Polytetradecene is the first one to be identify in the sample 2 and doesn’t have their presence on the previous sample 1. Polytetradecene was the microplastic is a synthetic polymer that are often used in lubricants, coatings, and adhesives as example of the product manufacture based on this polymer is cosmetics products such as lotion and creams that provide smooth and long last feel for a long time (Mepex, S., & Kappes, B., 2022).

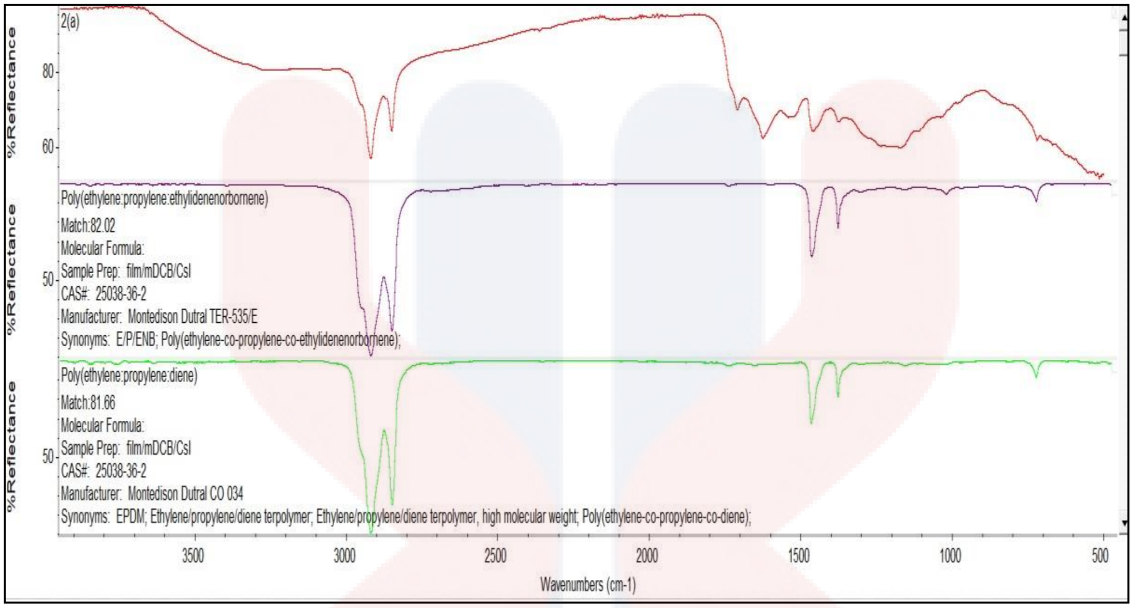


Figure 3.9: Results shows Polyethylene, Polyprophylene, PEN and Polyprophylene dene composition in stingless bee (replicate 1) from MR MADU KELULUT using FTIR

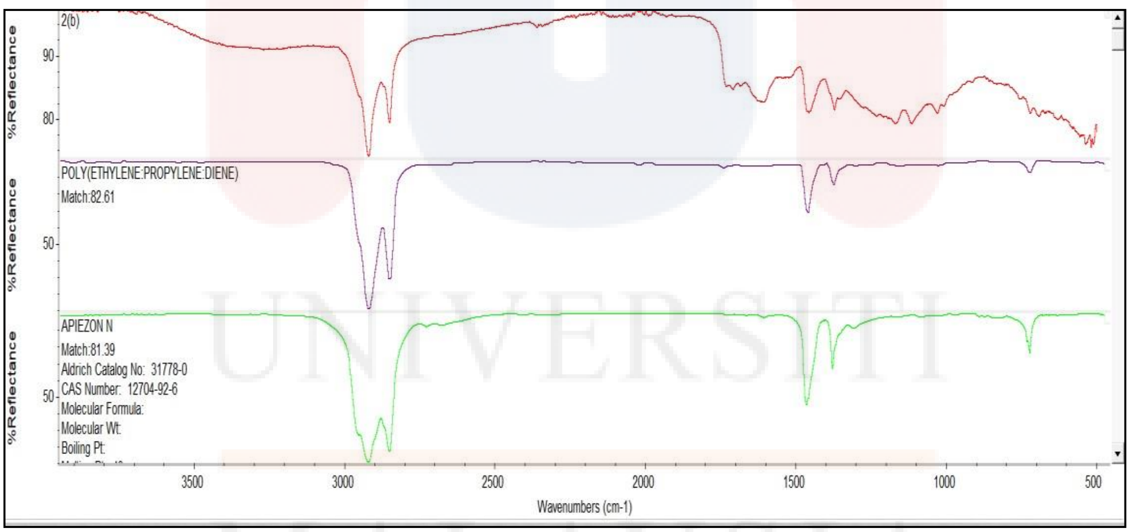


Figure 4: Results shows Polyethylene, Polyprophylene, PEN and Polyprophylene: Diene and Apiezon N composition in stingless bee (replicate 2) from MR MADU KELULUT using FTIR

While in the sample 2(c), there is another first difference microplastic presence others than previous findings which is Polyvinyl Chloride. 3 difference microplastic compound are being recognized under the FTIR analysis including polyvinyl chloride (57.4, 32%), polyethylene (59.06, 33%), and polydiene (64.39, 35%) that are being the highest microplastic found in the sample 2 (c). Polyvinyl chloride (PVC) needs to give more particular attention as it is a widely used plastic with various application but also potential environment concerns. PVC is commonly found in construction materials (pipes, window frames, and flooring), packaging and consumer products due to its durability, moisture resistance, and low cost. However, the presence of chlorine in PVC raises concerns about the potential that this microplastics can release toxic substances during its production use and disposal (Naggar, Y.A et al., 2021). Stingless bee may inadvertently ingest it and become exposed to these microplastics through contaminated nectar, pollen, water sources, ground surface, and including air. Exposures of this microplastic is caused by improper waste management and industrial areas since the local bee farm was nearby with factory industrial. PVC have potential can release harmful substance like phthalates and dioxins that can affect various organism including stingless bee as a pollinator indicator that reduce their health, reproductive and development of honey (Al-Kahtani, S. N. et al., 2021).

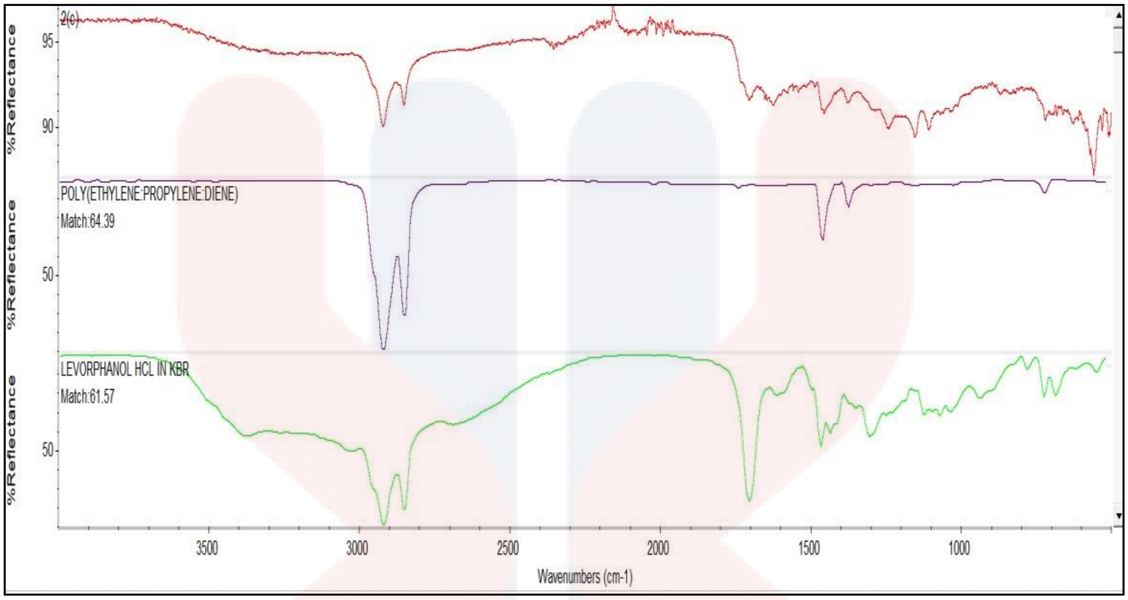


Figure 4.1: Results shows Polyethylene, Polyprophylene: Diene and Levorphanol HCL IN KBR composition in stingless bee (replicate 3) from MR MADU KELULUT using FTIR

Lastly represent from the sample 3 that have been collected on urban area local bee farm difference with sample 1 and sample 2 that only taken from the rural area local bee farm. The four types of microplastics shown in sample 3(a) are polyethylene, polydiene, polyethylidenornorbornene, and polytetradecene, difference with sample 3(b) and 3(c) that only have three type microplastic presence that have been mentioned in sample 3(a) except for polytetradecene. Comparing the three sample 3 through the result from pie chart, sample 3(c) have recorded the highest percentage of microplastic occur which is polyethylene type (83.23, 33%) and polydiene (84.62, 34%) compared to the sample 3(a) and 3(b). Even the stingless bee sample in 3(c) doesn’t occur any presence of microplastic detection from their outer body or morphology, they still manage resulting to became the highest amount of these two kind microplastic type compared to the others two sample replicate. Factors of these contaminate microplastic inside the stingless bee is regarding to urban area are mostly easily area that getting expose with microplastic pollutant because of industrial and waste management area, additional that microplastics also can travel through air, water, animal, and human itself as a transportation over long distance to their distribution in remote areas (Blasing, M., & Amelung, W., 2018).

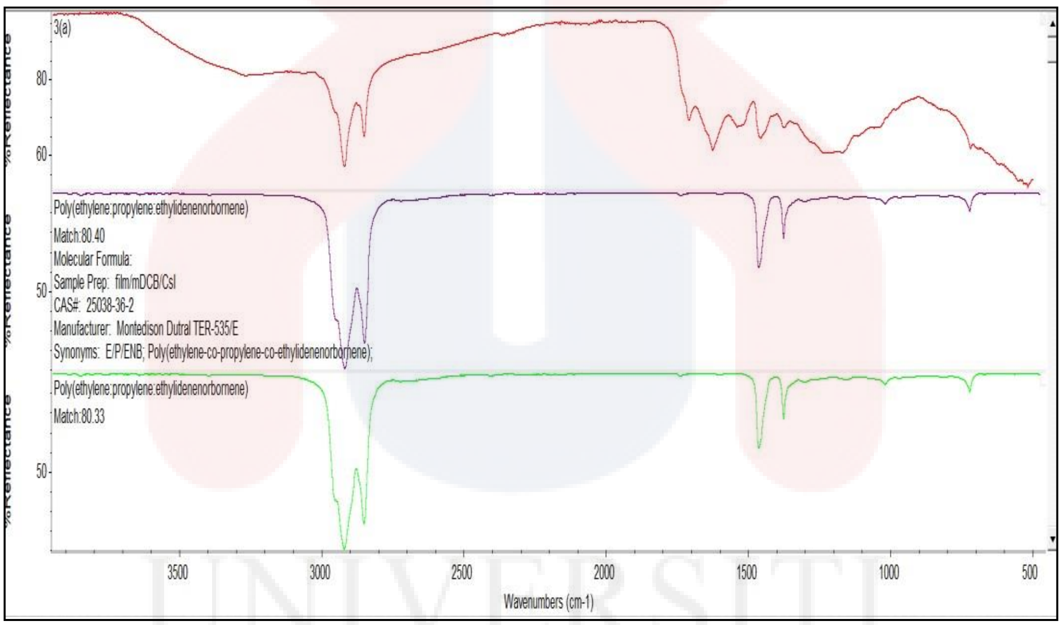


Figure 4.2: Results shows Polyethylene, Polyprophylene, and PEN composition in stingless bee (replicate 1) from Madu Kelulut using FTIR

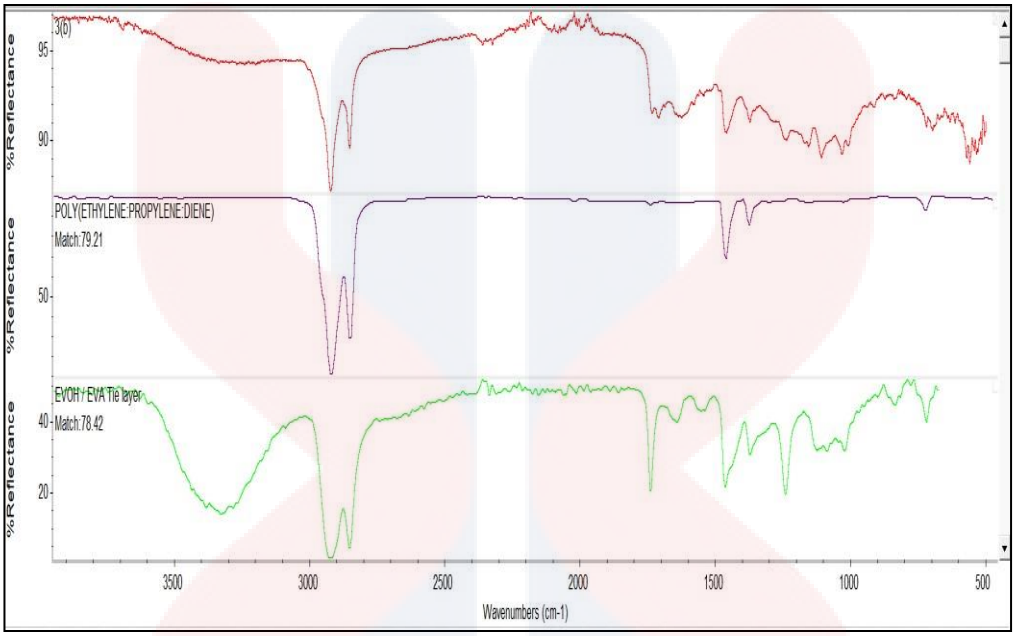


Figure 4.3: Results shows Polyethylene, Polyprophylene: Diene, and Evoh/Eva Tie layer composition in stingless bee (replicate 2) from Madu Kelulut using FTIR

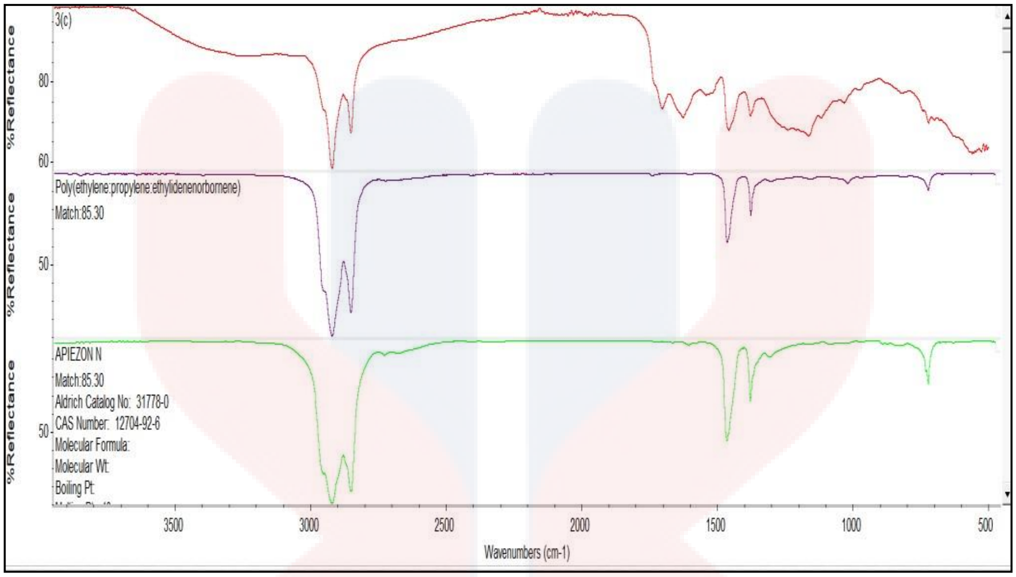


Figure 4.4: Results shows Polyethylene, Polyprophylene, PEN, and Apiezon N composition in stingless bee (replicate 3) from Madu Kelulut using FTIR

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusions

From this comprehensive study has shed light on the alarming presence of microplastics within stingless bee, through the quantitative advanced analytical techniques that applied, researchers are able to determined successfully the occurrence and characterized the types of microplastics found in various matrices associated within stingless bee. The detection of microplastic particles including fibers and films in the bodies of stingless bee. The findings of this study raise significant concerns about the potential impacts of microplastic ingestion on stingless bee health, while the full extent of these effects requires further investigation. The presence of microplastics within these crucial pollinators highlights the urgency of addressing these emerging environmental issues.

Moreover, research has demonstrated the potential of stingless bees serve as bioindicators of microplastic pollution in terrestrial ecosystem. Their ability to accumulate and reflect microplastic contamination levels in their environment makes them valuable sentinels for monitoring and accessing the extent of microplastic pollution. Moving forward, it is imperative to expand research efforts on this kind of studies to understand the specific mechanisms and long-term consequences of microplastic exposure on stingless bee physiology, behavior, and reproduction. Additionally, exploring the potential for microplastics to transfer through the food chain and accumulate in higher trophic levels is essential for comprehending the broader ecological implications.

The findings of this study underscore the immediate need of action and collaboration among researchers, any others research centre agency, and stakeholders to develop effective mitigation strategies and implement measures to reduce microplastic pollution. Protecting stingless bee and their vital ecosystem services is crucial for maintaining the delicate balance of terrestrial ecosystems and ensuring long-term environmental sustainability. In conclusion, this research contributed to the growing knowledge on the pervasiveness of microplastics and their potential impacts on critical species like stingless bee. It serves as a call to action for further exploration, awareness, and collaborative efforts to address this emerging environmental challenge and safeguard the health of our ecosystems for future generations.

5.2 Recommendations

There is a recommendation strategies and improvement that can be suggested. By expanding the monitoring and research efforts, through conduct long-term monitoring studies are supporting to track the spatial and temporal distribution of microplastics in stingless bee and their habitats. Investigate the specific mechanisms of microplastic uptake, accumulation, and potential trophic transfer in stingless bee food chains. Through the monitoring also, researcher can explore the potential for microplastics to act as vectors for the transport of pollutants, pathogens, and chemicals, and posing additional risks to stingless bee.

Developed standardized methodologies are also recommended, by establish the standardized protocols for sampling, processing, and analyzing microplastics on stingless bee and their surrounding environment. Through collaborate with international organizations and research institutions to harmonize methodologies and facilitate cross-study comparisons data sharing. Implement mitigation and preventive measures, promote the use of biodegradable and environmentally friendly alternatives to conventional plastics in various sectors such as agriculture, packaging, and consumer products. Encouraging the proper waste management practices including recycling and responsible disposal to reduce the release of microplastics into the environment, followed with support the clear-up initiatives and remediation efforts in areas with high microplastics contamination particularly near stingless bee habitats.

Further strategies that can be suggested is strengthen the conservation efforts, starting with prioritize the protection and restoration of stingless bee habitats to reduce their exposure to microplastic pollution and any other environmental stressors. Based on what kind of microplastic are resulted can be found on stingless bee, we can implement sustainable land management practices that minimize the release of microplastic in terrestrial ecosystems. Otherwise, we can develop and promote the use of stingless bee as bioindicator in environmental monitoring programs and risk assessment studies.

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APPENDIX A

	A	B	C	D	E	F	G	H	I	J	K	L
1		Polysulfone	Polyarylsulfone	Polyarylether	Polyethersulfone	Polyprophylene	Polyethylene	Polydiene	Polyethylidenenorbornene	Polytetradecene	Polyvinyl Chloride	
2												
3	1(a)	63.57	62.32	59.62	61.22							
4	1(b)					78.12	68.52					
5	1(c)							82.06	78.39			
6												
7	2(a)						82.02	81.29		80.13		
8	2(b)						82.61	81.32		80.28		
9	2(c)						59.06	64.39			57.4	
10												
11	3(a)						78.67	79.77	80.4	79.06		
12	3(b)						73.72	79.21	73.5			
13	3(c)						83.23	83.45	84.62			

Data gain from FTIR and OMNIC software reading insert in an Excel to display the bar chart and pie chart for the results

APPENDIX B

Sample collection on local bee farm with bee farm owners help

Observe and isolate the microplastic from stingless bee morphology

Digestion process using Hydrochloric acid 30% (HCL) with Sodium chloride (NaCL) applied with safety procedure followed using Laminar flow cabinet

Filtration process using vacuum pump to separate or filter the microplastic and chemical substance that have been used