

Article

# Detection of Microplastic Contamination in Commercial Insect Meals

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**Abstract:** Background: Escalating global plastic production, expected to reach 34,000 million tons by 2050, poses a significant threat to human and environmental well-being, particularly in aquatic ecosystems. Microplastics (MP) and nanoplastics (NP), which originate from the degradation of plastics, are of concern due to their potential bioaccumulation and uptake of pollutants. This study addresses identification methods and focuses on insect meal, a raw material for aquaculture feed. Methods: By using different techniques, the study was able to detect MP and NP in insect meal samples. Chemical digestion with KOH at 60 °C efficiently removed organic matter without affecting the synthetic polymer polyethylene (PE). Filtration, confocal Raman microscopy, SEM, and TEM were used for comprehensive analysis, and integrity tests on PE films were performed using Raman and FTIR spectroscopy. The results showed the presence of PE microplastic particles in the insect meal, which was confirmed by correlative Raman and SEM mapping on a positively charged surface. In addition, the increased resolution of the Raman microscope identified submicrometric PE NP (800 nm). Transmission electron microscopy (TEM) with energy-dispersive X-ray spectroscopy (EDX) confirmed plastic-like structures in the insect meal, highlighting the presence of PE plastics characterized by irregular shapes and some agglomeration. The higher carbon concentration in the EDX analysis supported the plastic nature, which was also confirmed by Raman spectroscopy. Conclusions: The study provides a robust method for the detection of MP and NP in insect meal and provides valuable insight into the possible presence of plastics in insect-based aquafeeds. The combination of different analytical methods increases the reliability of the results and sets the stage for future investigations that could focus on the quantification of NP and the assessment of their potential environmental impact.

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## 1. Introduction

Global plastics production amounted to 359 million tons in 2018 and is estimated to increase to 34,000 million tons by 2050 [1]. Plastics are a large class of synthetic or semi-synthetic organic polymers with a high molecular weight. The most commonly produced polymers are polyethylene (PE), polypropylene (PP), and polystyrene (PS), which are also among the most common plastics polluting the aquatic environment [2]. Plastic pollution

is now considered the greatest threat to human and environmental health, especially to the aquatic environment in which they accumulate [3,4]. The risk is exacerbated by the progressive fragmentation of these plastic wastes in the environmental media caused by physical, chemical, and biological processes or a combination of these processes, such as thermal degradation, oxidative degradation, hydrolysis, biodegradation, UV photodegradation, corrosion, and mechanical abrasion [5,6].

Microplastics (MP) are defined as plastic particles with a diameter of 1  $\mu\text{m}$  to 1 mm, while nanoplastics (NP) have a size of 1–1000 nm [7], although the definition of the term NP is still controversial. Based on the source of origin, MP are categorized into primary MP, which are intentionally produced for specific commercial purposes, and secondary MP, which result from the decomposition of larger plastic parts. The latter are predominant in the environment and make up about 80% of the plastic waste found [8]. Secondary MP are ubiquitous in aquatic and terrestrial environments [9,10] and tend to be irregular in size, shape, and composition, contributing to an increased potential risk to organisms.

The risks posed by MP and their nanoscale forms include their bioaccumulation along the food chain [11] and their ability to absorb and concentrate hydrophobic chemical pollutants, such as pesticides or pharmaceuticals [12–14]. In aquatic vertebrates, MP accumulates mainly in the digestive system and causes various adverse effects, such as gastrointestinal (GI) tract damage, alterations in lipid metabolism, behavioural changes, cytotoxicity, and dysbiosis [15–22]. In addition, the transfer of MP from feed to liver and fillet has been described in two of the most important commercial marine fish species for the Mediterranean region, namely, gilthead seabream (*Sparus aurata*) [23] and European seabass (*Dicentrarchus labrax*) [24].

As a possible negative impact on the safety of seafood and a potential risk to human health cannot be excluded, the identification of MP is becoming an increasingly important issue for consumer health. The official EU limit for plastics in animal feed is zero, although many countries actually work with 0.15%. On the other hand, the European Food Safety Authority (EFSA) has acknowledged that there is no legislation for MP and NP as contaminants in food and that reliable identification methods need to be developed, especially for smaller MP that are more likely to cross the intestinal barrier.

The status of currently applied identification and quantification methods for MP and NP has been reported in several reviews [1,25–29] and a new analytical method for NP in complex matrices rich in organic material has been developed [30].

There are currently no uniform standard methods for the characterization and detection of MP. An analytical protocol for the detection and characterization of MPs and NPs usually requires three main steps: extraction from the matrix; separation, quantification, and sizing; and characterization and/or identification of plastic particles [1,26,31].

Raman and Fourier-transform infrared spectroscopy (FTIR) are the most commonly used techniques to identify plastic particles in studies on microplastics. Both techniques have the advantage that they are non-destructive and require only a small amount of sample. However, compared to FTIR spectroscopy, Raman techniques have higher resolution (up to 1  $\mu\text{m}$ , while FTIR resolution is 10–20  $\mu\text{m}$ ), broader spectral coverage, higher sensitivity to non-polar functional groups, lower water interference, and narrower spectral bands. In combination with a microscope, micro-Raman spectroscopy ( $\mu$ -Raman) can characterize MP in the 1–10  $\mu\text{m}$  size range that are otherwise undetectable with FTIR [1,27]. On the other hand, the detection time with Raman imaging is significantly higher than with FTIR imaging.

Recently, a novel approach to isolating NPs from mussels and subsequent sampling by  $\mu$ -Raman analysis has proven successful [30]. Instead, rapid qualitative discrimination between polymers, metals, and inorganic particles at the nanoscale could be performed using a scanning electron microscope (SEM) or transmission electron microscopy (TEM) in combination with energy-dispersive X-ray spectroscopy (EDX) [32].

There is very recent evidence of microplastics in raw materials used to produce fish feed [33]. For example, Thiele and colleagues [34] found MP contamination in commercial

samples of fishmeal, a product derived mainly from whole wild-caught fish or bycatch or by-products of marine fisheries. Fishmeal is a highly valued nutrient source used as a raw material for the production of feed for farmed terrestrial and aquatic animals, including fish and shrimp. In fact, about 70% of the demand for fishmeal currently comes from aquaculture, which supplies nearly 50% of the seafood consumed by humans. According to Thiele and colleagues [34], who reviewed the literature up to 2021 on MP in two major marine fish species (whitefish and sardine/anchovy) used for fishmeal production and critically examined the methods used for MP detection, commercial samples contained  $123.9 \pm 16.5$  MP/kg of fishmeal—mainly polyethylene—and  $52.0 \pm 14.0$  microfibres—mainly rayon. MP concentrations in processed fishmeal appear to be higher than in caught fish, suggesting a possible increase during the production process [34].

However, most wild catches are at or above the maximum sustainable yield. As a result, aquaculture can no longer rely on marine resources to produce fish feed, and such feed options are simply not sustainable. To avert ecological damage and cover rising costs, fish farmers and commercial feed manufacturers have made significant efforts to reduce the amount of fishmeal in aquaculture feed by replacing it with other protein sources. In this respect, insects could become a major player in the fish feed market, and insect larval meal could become a sustainable and commercially viable alternative to fishmeal in aquaculture. In particular, the black soldier fly, *Hermetia illucens*, has emerged as one of the most promising insect species that can be used in aquafeeds as an alternative protein source to replace fishmeal, as the meal of *H. illucens* is rich in proteins (45–75% dry matter), essential amino acids, lipids, minerals, and vitamins [35–39].

The insect farming industry can be seen as a model for the circular economy, as commonly available organic waste, mainly agricultural and food waste, is successfully used on an industrial scale to produce BSF larvae. However, as Regulation (EC) No. 1069/2009 stipulates that insects kept in the EU for food, feed, or other purposes are “farmed animals”, it is prohibited to feed insects with faeces or separated contents of the digestive tract, manure or food waste, and processed animal proteins, with the exception of fishmeal (Regulation (EC) Nos. 767/2009 and 999/2001). Considering that insect farming is a regenerative system, where the by-products of one process serve as raw material for another process, and in perfect accordance with the circular economy model, we can assume that MP and NP enter the feed chain and eventually return to our diet through the consumption of fish.

The contamination of insect meal with MP and NP can occur at various stages of the production process, both when the insects are fed and when they are processed into meal.

Indeed, during the rearing of insects, the feed they are given can contain MP. This can happen if the feed itself is contaminated with MP or if the environment from which the feed originates is polluted with MP. Insects can ingest MP and NP along with their food, and these particles can accumulate in their bodies over time.

MP can also contaminate insect meal during the processing of insects into meal. This can happen through various mechanisms:

- Mechanical contamination: microplastics present in the environment where insects are processed (e.g., air, surfaces, and equipment) can stick to the insects or contaminate the processing machinery, causing them to end up in the final meal product.
- Cross-contamination: if the equipment or facilities used to process insect meal are not properly cleaned and maintained, there is a risk of cross-contamination from other sources of microplastics present in the processing environment.
- Packaging and storage: improper packaging materials or storage conditions can introduce microplastics into the insect meal during transport and storage.

Overall, both the feeding of insects and the processing of insects into meals offer the possibility of contamination with MP. To ensure the safety and quality of insect meal as a sustainable source of protein, effective measures to control MP and NP contamination at every stage of production are essential.

In this study, we demonstrate a method for the detection and identification of MP and NP in a commercial raw material, such as *H. illucens* insect larvae meal is used for the production of aqua feed. To our knowledge, this matrix has not yet been investigated in any study. To evaluate the presence of plastics in insect meal,  $\mu$ -Raman electron microscopy (SEM and TEM) was used for the detection of MP and NP in complex matrices according to our previously proposed analytical protocol [30,40].

## 2. Materials and Methods

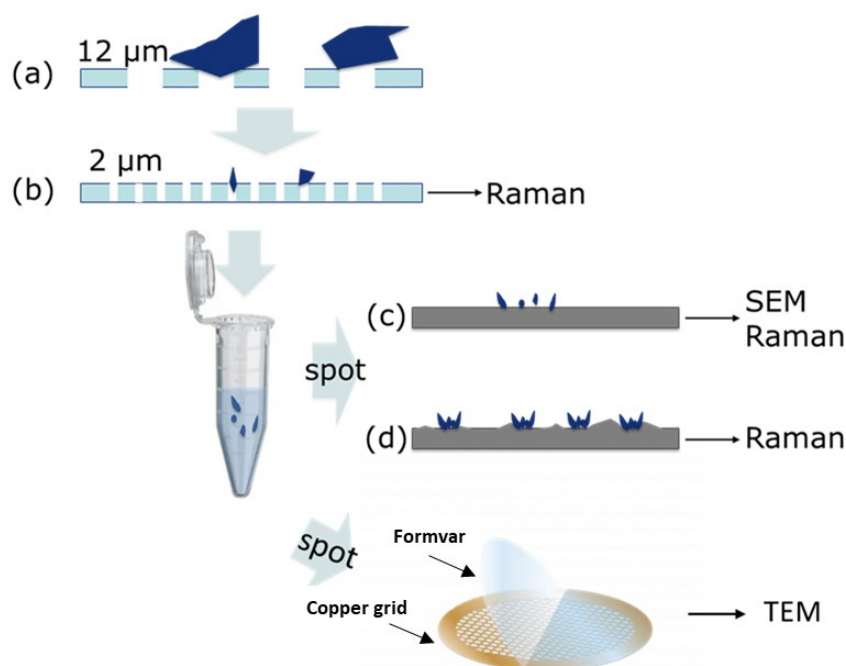
### 2.1. Chemical Digestion with KOH

An aliquot (0.5 g) of commercially available insect meal was digested overnight with 10 mL of 10% KOH (w/vol) at 60 °C with mechanical shaking in 15 mL conical glass centrifuge tubes. The digested samples were stored at 4 °C until the planned analyses.

### 2.2. Filtration of the Sample

The scheme for the detection of “small” MP and NP in insect meal samples is shown in Figure 1.

One millilitre of the digested sample was diluted 1:1 in MilliQ water. Two consecutive vacuum filtration steps were performed on 25 mm diameter polycarbonate filter membranes (Whatman® Nuclepore™ Track-Etched membranes, Cleves, OH, USA). The first filtration was performed with a filter pore size of 12  $\mu$ m. The eluate was then collected and filtered again on a filter with a pore size of 2  $\mu$ m. After washing with 1 mL of MilliQ water, the filter and filtrate (approximately 2 mL) were stored at +4 °C until analysis. In parallel, 1 mL of MilliQ water was filtered as a negative control.



**Figure 1.** Scheme for the detection of “small” MP and NP in fishmeal samples. (a,b) filtration step; (c) aliquot of filtrate spotted onto a Teflon-coated silicon wafer; (d) aliquot of filtrate spotted onto superhydrophobic surface.

### 2.3. Confocal Raman Microscopy (CRM) Analysis of 2–12 $\mu$ m Plastic Fraction

The filters were analysed with the Via™ Confocal Raman microscope (Renishaw, Wotton-under-Edge, UK). Objects were recognized by manual point-by-point mapping. The optimization of the Raman signal was set as follows: 1 s, 10% laser, 10 accumulations, range of 1700–3000  $\text{cm}^{-1}$ . An extended analysis of the spectrum (200–3500  $\text{cm}^{-1}$ ) was performed for the most interesting objects by setting the Raman signal to 5 s, 10% laser, and 5 accumulations. The materials were identified after baseline subtraction of the acquired raw spectra using WiRE 5.5 software (Windows-based Raman environment) and a self-created polymer database. Renishaw's WiRE software is specially tailored to Raman spectroscopy. It controls the acquisition of the spectra and offers a whole range of data processing and analysis functions. Open Specy, an open-source spectrum library, was used to identify the polymer type of MP [41].

#### 2.4. Scanning Electron Microscopy (SEM) Analysis of <12 $\mu\text{m}$ and <2 $\mu\text{m}$ Plastic Fractions

One millilitre of the digested sample, diluted at 1:1 in MilliQ water, was filtered onto polycarbonate membranes with a pore size of 12  $\mu\text{m}$  (Whatman® Nuclepore™ track-etched membranes); the filtrates were recovered and spread onto a Teflon-coated silicon chip by immersion for 30 min. The silicon chips were then dried under nitrogen. Alternatively, 200  $\mu\text{L}$  of the eluate was centrifuged at maximum speed for 5 min, the supernatant was discarded, and the resulting pellet was washed with 500  $\mu\text{L}$  of phosphate buffer (PB, 10 mM) and resuspended in 20  $\mu\text{L}$  of PB (10 mM). Samples were then manually spotted onto a Teflon-coated silicon (Si) wafer (1  $\mu\text{L}$ ) and dried under nitrogen flow prior to imaging.

For the analysis of plastic particles smaller than 2  $\mu\text{m}$ , the filtrate of a filter with a pore size of 2  $\mu\text{m}$  (approximately 2 mL) was concentrated to 200  $\mu\text{L}$  using an Amicon Ultra 2 mL centrifugal filter unit (30,000 MWCO, Merk-Millipore, Burlington, MA, USA). The centrifugal filter unit was washed three times with 1 mL of MilliQ water. The sample was then loaded onto the filter unit and centrifuged at 4000 $\times$  g for 3 min or until the volume was halved at room temperature. One mL of PB buffer (10 mM) was then added to the Amicon filter unit, which was again centrifuged at 4000 $\times$  g for 3 min. This step was repeated twice to replace the KOH solution with PB buffer. Finally, the filter unit was centrifuged for as long as necessary to concentrate the sample to 200  $\mu\text{L}$ . The concentrated sample was recovered by spinning backwards at 1000 $\times$  g for 1 min.

To induce the aggregation of plastic particles, one microliter of the concentrate was manually spotted onto a superhydrophobic surface and dried as described in Valsesia et al. [30]. To evaluate the presence and distribution of small plastic particles, 10  $\mu\text{L}$  of the sample was spotted on a positively charged surface to immobilize the particles in randomly separated positions and allow SEM counting and Raman identification. The preparation of the Si wafer surface was described in detail in [42]. In brief, the Si surface was first coated with a plasma-coated hydrophobic layer of polytetrafluoroethylene and then incubated alternately with positively charged polydiallyldimethylammonium chloride or negatively charged poly (sodium 4-styrenesulfonate) to increase the hydrophilic character of the surface.

Scanning electron microscopy of the samples was performed using a Nova 600i Nanolab (Termofisher, Eindhoven, The Netherlands) equipped with an EDX system for elemental analysis (EDAX Inc., Mahwah, NJ, USA).

#### 2.5. Transmission Electron Microscopy (TEM) Analysis of <2 $\mu\text{m}$ Plastic Fraction

TEM (JEOL JEM-2100, JEOL, Basiglio, Italy) in conjunction with EDX (Brüker, Milan, Italy) was used at 120 kV in both TEM and STEM modes to characterize the primary size, morphology, and elemental composition of plastic-like particles at the nanoscale.

A digested insect meal sample (3  $\mu\text{L}$  of the sample suspension) was applied to a 200 mesh Formvar (Agar Scientific, Redding, CA, USA) carbon-coated copper grid and dried overnight in a desiccator. The grid was then washed twice with MilliQ water, dried, and analysed.

Elemental analysis was performed in STEM, brightfield, and hypermap modes (Quantax software, Brüker, Milan, Italy) to determine the carbon content.

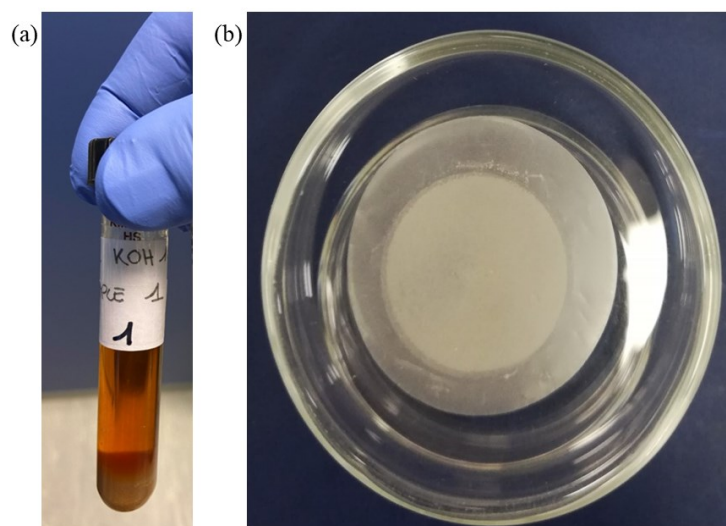
## 2.6. Microplastics Integrity Test

As a control, 1 mm thick PE films (Sigma Aldrich, Milan, Italy) were cut into small pieces of 5–6 mm in size. Each PE piece was weighed to the nearest 0.1 µg (Mettler Toledo® XPR2U Ultra-Micro Balance, Columbus, OH, USA) and placed in a glass vial containing 7 mL of 10% (w/vol) KOH and water. The suspension was allowed to stand overnight at 60 °C with mechanical shaking and then stored at RT and in the refrigerator (4 °C) for 3 weeks. Each PE piece was then rinsed in water and dried at 40 °C for one week until the weight was constant. The PE pieces were then weighed to determine any variations in weight. The PE pieces were also analysed using Raman and FTIR spectroscopy to scan the test samples and observe the chemical properties.

## 3. Results

### 3.1. KOH Digestion

KOH treatment at 60 °C efficiently digested the organic matter of the lipid-rich insect meal sample and removed a large part of the biological matrix (Figure 2), while the synthetic target polymer polyethylene (PE) remained intact. The resistance test of PE to the applied digestion protocol showed no significant change in PE unit weight and no changes in chemical structure after KOH treatment (Supplementary Materials File S1).



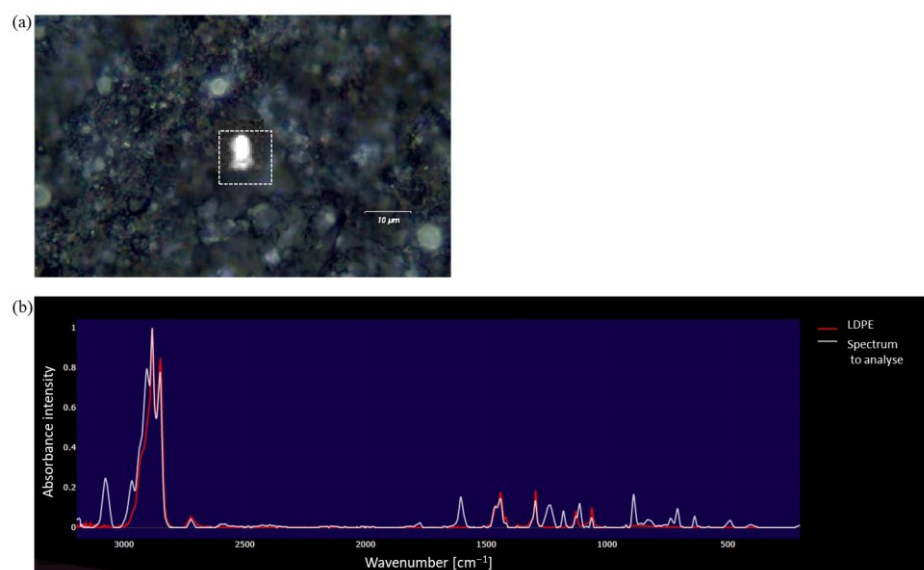
**Figure 2.** Insect meal sample after (a) digestion with potassium hydroxide (KOH) at 60 °C, and (b) filtration on a polycarbonate filter with a 2 µm pore size.

### 3.2. Raman Mapping and Analysis of Measured Spectra of the 2 µm Filter

Six different areas of the filter (0.095 mm × 0.060 mm), corresponding to 0.01% of the total filter area, were analysed using Renishaw CRM. A total of 328 objects were manually detected and mapped using a 100X objective. The spectrum (range: 1700–3000 cm<sup>-1</sup>) of seven objectives was recognized as polymer origin. More precisely, these were PE MPs with a size of <10 µm (Figure 3). Considering the volume of the sample analysed on the filter (1 mL, 10% of the total volume) and the area fraction of the filter analysed at the best resolution, the potential hit of the polymer objects in the whole sample was:

$$7/0.1 \times 0.0001 = 7 \times 10^5 \text{ particles/10 mL or } 7 \times 10^5 \text{ particles/0.5 g}$$

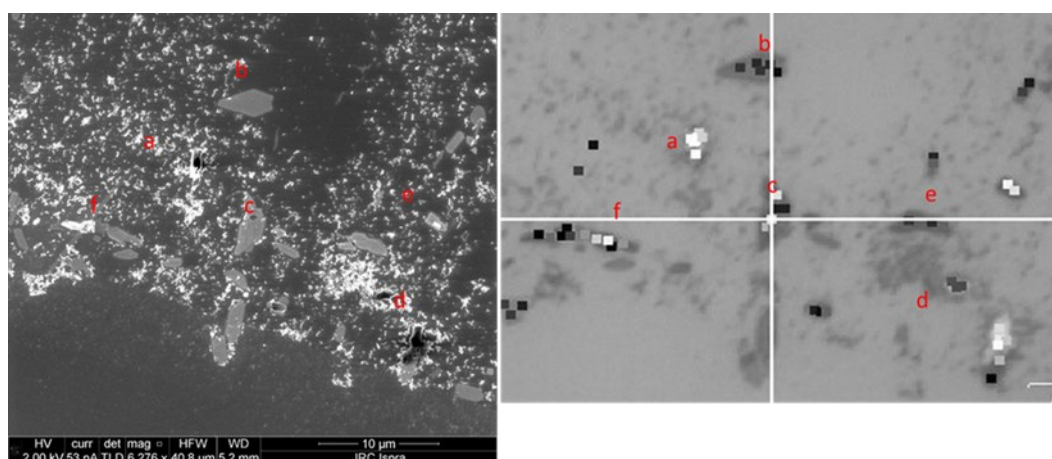




**Figure 3.** Identification of MP extracted from insect meal using CRM: (a) Raman map image of a single particle (dotted) and its relative confocal Raman spectrum, and (b) the spectrum of the selected object (white) was compared with the original reference spectrum (red). (Open Specy v0.9.3).

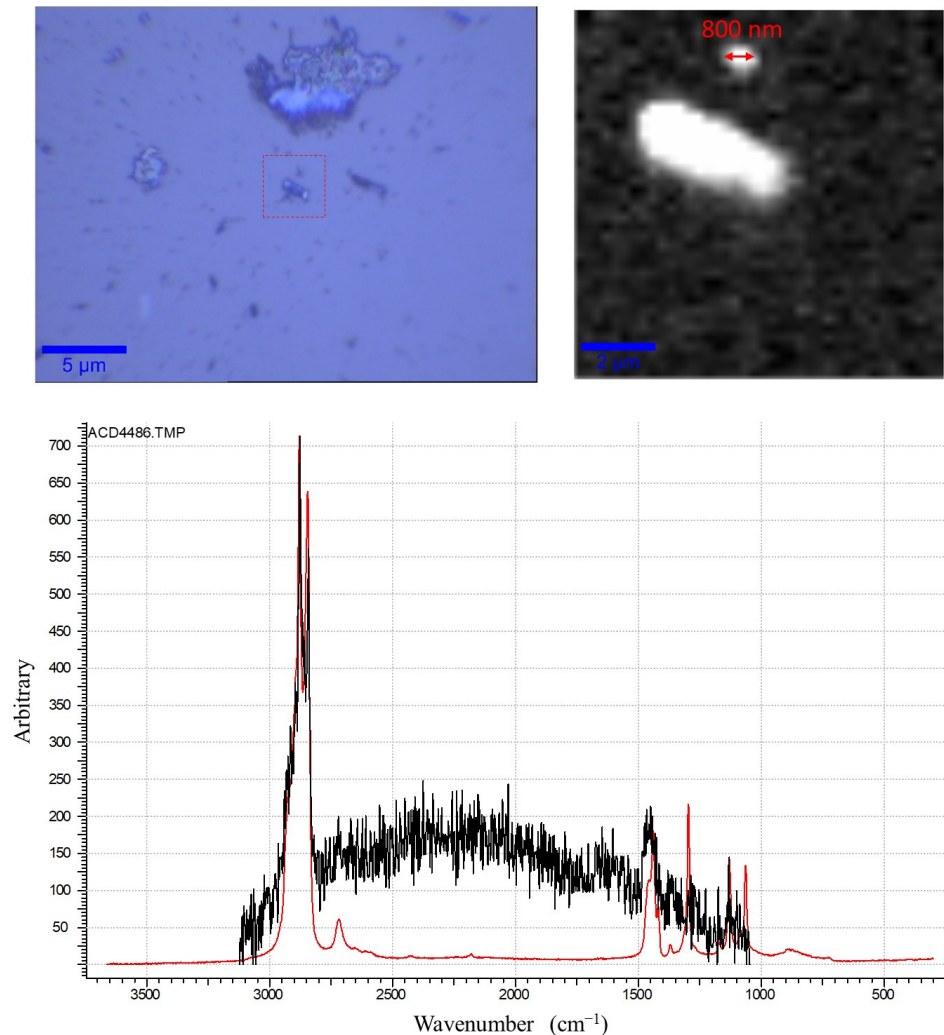
### 3.3. Correlative Raman and SEM Mapping and Analysis of Measured Spectra of the Particulate <math>< 2 \mu\text{m}</math> on a Positively Charged Surface

The particles that passed through the filter with a filtration fineness of 2 μm were then concentrated on a positively charged surface, allowing correlative Raman and SEM mapping. A particularly particle-rich area was selected with the SEM, and the particles were recognized by their shape and size. After e-beam irradiation, the area appeared brighter in the optical microscope connected to the confocal Raman microspectrometer. In this way, the same area that was analysed in SEM was detected in the optical microscope, and the particles were labelled with the same letter in the two images. The Raman spectrum of each object was then captured and recorded. A comparative analysis of the probability of the spectra with the spectrum of PE was performed by the software of the device. The software then classified the particles with a colour scale: “white” for particles (or dots) whose spectrum is practically identical to that of PE and “black” for spectra that are not similar to that of PE. The results are shown in Figure 4. The particles labelled a, f, d, and b were identified as PE. Other particles similar in shape and morphology were not classified as PE.



**Figure 4.** SEM-RAMAN comparison: The white dots in the Raman correspond to a good match with the PE spectrum, the grey dots correspond to a partial match and the black dots indicate no match with the PE reference spectrum. The identified PE particles are labelled with red letters.

By increasing the resolution of the Raman microscope with a 100X objective, an area around a selected particle was scanned with the highest possible scan resolution (X and Y steps of 100 nm). In this way, a submicrometric PE particle characterized by a size of 800 nm was identified and mapped (Figure 5). This result proves the presence of PE NP in the sample.

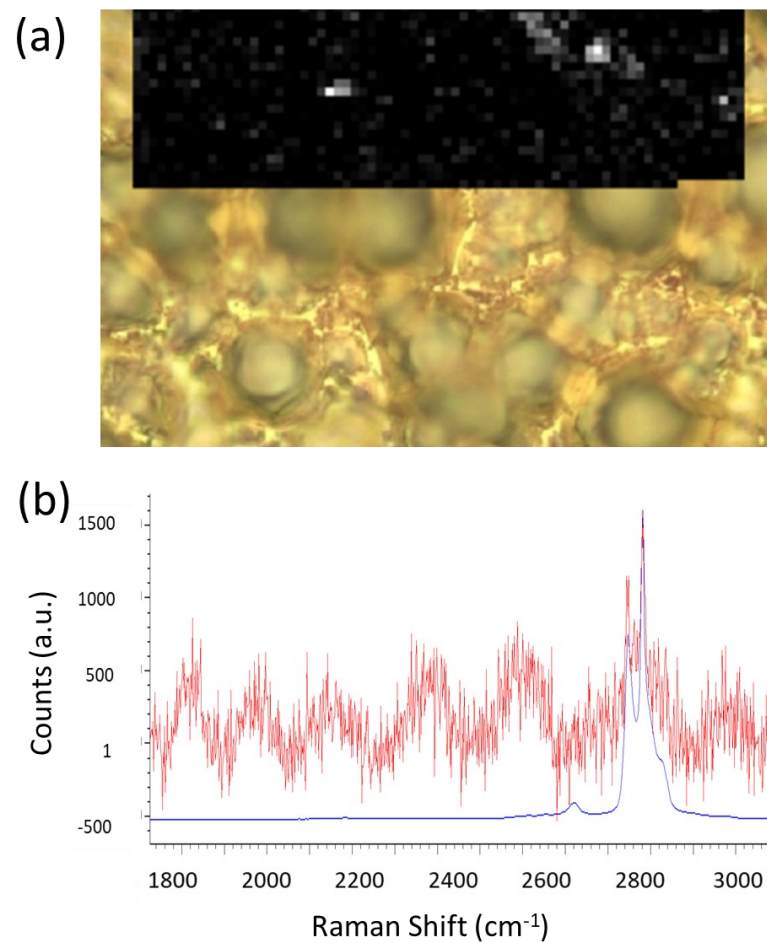


**Figure 5.** High-resolution Raman map of a few particles on the T5 surface. NP (<1 µm) were detected. **Top-left:** optical image of the analysed area. The red square is the imaged area by the Raman microscope. **Top-right:** Raman intensity map for the peak at 2900 cm<sup>-1</sup> corresponding to the signal from the PE. **Bottom:** black curve: typical spectrum of the white areas in Figure 5 top-right. Red curve: PE reference spectrum.

### 3.4. Raman Mapping and Analysis of Measured Spectra of the Particulate <2 µm Spotted on a Superhydrophobic Surface

With this approach, small NPs were clustered on the superhydrophobic surface and mapped with Raman. A map with a resolution of <100 nm was acquired with a 100X objective. Again, the white pixels were associated with a spectrum similar to the PE spectrum, while the black pixels were not recognized as PE. Different PE clusters were recognized by the software (Figure 6).



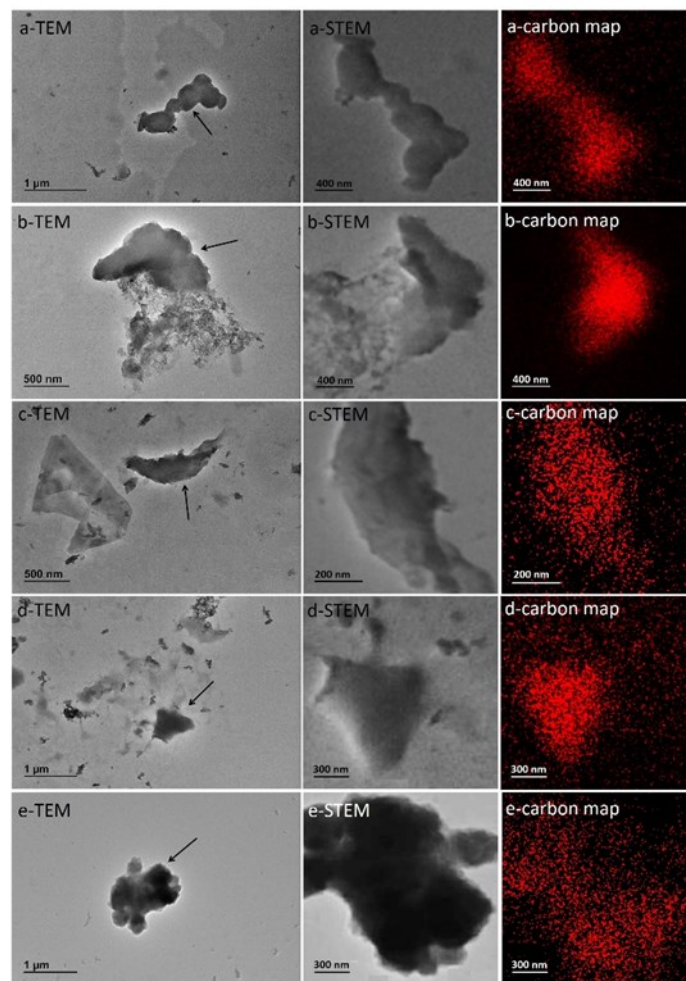


**Figure 6.** Sample filtered  $<2\ \mu\text{m}$  (NP fraction) and blotted over a superhydrophobic surface to cause aggregation of the particles. **(a)** Raman intensity map (black and white) superimposed to the optical image of the surface. The white areas correspond to the PE-like spectrum, hence to the presence of PE particles. **(b)** The red line is a typical Raman spectrum of the white areas in Figure 6a. The blue line corresponds to the reference spectra for PE.

### 3.5. TEM Characterization of MPs

TEM in conjunction with EDX confirmed the presence of plastic-like morphological structures as observed by SEM and confocal Raman microscopy in the digested insect meal sample. Sample preparation allowed sufficient matrix to be removed to see whole particles approximately  $1\ \mu\text{m}$  in size and below, which were irregularly shaped (Figure 7) and in some cases agglomerated/aggregated (Figure 7a,e). The corresponding EDX analysis in STEM mode showed that the objects found contained carbon in a higher concentration than the background.

This finding suggests that they could be PE plastics, which was confirmed by Raman spectroscopy.



**Figure 7.** Electron microscopic analysis of the suspension of digested insect meal. Images taken in TEM and STEM modes and the corresponding carbon map analysis are reported. Representative TEM images of the sample contain a small amount of matrix under which particles can be seen (arrow). Both whole pieces (b,c,d) and agglomerates/aggregates (a,e) can be seen. It is possible to observe the heterogeneous shape and the irregular edges of the MP. The STEM images are analysed with EDX and show the map of carbon distribution (red).

#### 4. Discussion

This study deals with the detection and identification of MP and NP in a commercial raw material, namely, insect larvae meal of *H. illucens*, which is used for the production of aquafeed. Various analytical techniques were used in this study, including Raman spectroscopy, correlative SEM-Raman imaging, and detection on a superhydrophobic surface.

Plastic pollution is a global problem, and there is an urgent need for effective detection methods due to the increasing production and environmental risks associated with plastics. The risks posed by MP and NP include bioaccumulation, absorption of pollutants, and the potential impact on aquatic organisms, highlighting the implications for seafood safety and human health.

MP and NP can enter the environment from various sources, and insects, like other animals, can be exposed to them through different routes, such as airborne deposition, soil contamination, and consumption of contaminated prey [43].

Synthetic textiles are the main source of airborne MP. Other sources include tire and brake particles from vehicles, the degradation of large plastics, and industrial emissions.

The size range of airborne MP ranges from 5000 nm to <25 nm. These MPs can be transported through the air and deposited on surfaces, including plants that insects feed on. This can occur through atmospheric processes such as wind and precipitation [43].

MP can also occur in soil, either directly through the dispersal of plastic waste or indirectly through the decomposition of larger plastic pieces into smaller particles over time. After rain and snowfall, rainwater can infiltrate the soil, potentially contributing to MP in the soil. Insects can ingest MP when they feed on contaminated food sources, for example, plants, other insects, or organic material containing microplastics.

MP can enter the bodies of insects by ingestion, inhalation, or skin contact. The uptake dose of MP in insects can vary greatly depending on species, life stage, and environmental conditions. Studies have shown that the concentration of MP in organisms can range from negligible amounts to significant quantities [43–46]. Insects pass these on to other animals when they themselves are eaten.

In recent years, this risk has become an increasingly pressing issue with the growing use of insect meal in animal feed. Indeed, aquafeed manufacturers have successfully incorporated insect proteins and oils into their formulations, reducing the use of fishmeal and fish oil while improving the sustainability and nutritional value of the feeds.

Therefore, it is crucial to find valid analytical methods for the detection of MP in complex matrices, such as animal feeds and the raw materials used for their formulation.

The main problem in the analysis is the isolation and detection of small plastic fragments in an extremely complex matrix, such as insect larvae meal. In this study, we approached the problem step-by-step. The first step was to simplify the solid matrix. To convert the solid matrix into a liquid dispersion, the KOH digestion strategy was applied. KOH is a general digestion method that also preserves the chemical integrity of the various plastic fragments. The result of KOH digestion is again an extremely complex matrix containing dissolved chemicals and colloidal particles of different sizes, ranging from NP to particles several micrometres in size.

Since we were looking for a smaller fraction of MP, we filtered the suspension with a 12 µm mesh filter to retain the large particles and further simplify the matrix. Raman mapping of the filter allowed us to analyse thousands of objects and check if their spectrum matched the spectrum of one of the most abundant polymers. Seven particles in the selected area were attributed to PE. If we assume that the distribution in the volume of the digested liquid and on the surface of the filter is homogeneous, we can estimate the concentration of MP in the digested matrix and, consequently, per mass of the original sample.

A number of 1430 particles per gram of material was calculated. Interestingly, we found MP and NP in the filtrate. To detect the presence of “small” MP (with a size of less than 12 µm), we used the approach of electrostatic trapping of particles on a functionalized surface. This method is relatively simple but allows the almost complete simplification of the matrix and the isolation of the “hard” particles present in it. All colloidal particles floating in a suspension are subjected to different forces (according to the DLVO theory [47], which keeps the particles in a state of equilibrium and moving in the fluid by Brownian motion). When a surface comes into contact with the suspension, the same DLVO forces act between the particles and the surface, which can be attractive or repulsive depending on the properties of the colloidal particles and the surface. In the present case, we have introduced a positively charged surface capable of attracting negatively charged particles and immobilizing them. Moreover, dispersed molecules, micelles, and aggregates of the matrix (if they are negatively charged) are attracted to the surface, but they do not keep their shape when immobilized because they are not as mechanically stable as the hard colloidal particles (such as MP and NP). The rate of absorption depends on many factors, such as the concentration of the particles in suspension and the rate of diffusion. After a time, a number of particles are immobilized on the surface and distributed in a semi-ordered manner by the particle–particle repulsion forces.

With this method, it is possible to image the colloidal dispersion on the surface and spatially isolate the “hard” MP and NP from the rest of the matrix. The particles are then

examined individually using CRM and SEM. In this way, we were able to detect individual PE particles with a size of  $<1 \mu\text{m}$ , which is close to the detection limit for Raman microscopy. To overcome this inherent size limitation of the instrument and to be able to detect smaller particles, we used a different approach to immobilize the particles. By using a superhydrophobic surface, we favoured the self-aggregation of the particles by inducing the drying of a dispersion droplet. During the drying process, capillary forces tend to aggregate the particles into clusters, which are then detectable by CRM. Clusters typically range in size from a few micrometres to tens of micrometres and can be detected with CRM. In this way, we were able to identify clusters containing PE and detect the presence of NP particles in the original sample.

The results of the present study show that insect meal can be a source of contamination of farmed fish with MP, and it is therefore possible that MP enters the farmed fish via the feed. Using CRM, we estimated  $14 \times 10^5$  particles/g insect meal. If we assume that the proportion of insect meal in a commercial feed is usually no more than 10%, this figure corresponds hypothetically to  $140 \times 10^6$  MP particles/kg of feed. A value that is significantly higher than the previously documented values in commercial feeds for Asian stingray catfish (*Heteropneustes fossilis*), European seabass, and tilapia (*Oreochromis niloticus*), which ranged between 500 and 11,600 MP particles/kg fish feed [48–50]. The deviating results can be explained, on the one hand, by the properties and composition of the ingredients. These previous data refer to a commercial feed containing only fishmeal and vegetable raw materials. As shown in various studies, the MP content of fishmeal can have a range of values from 0 to 17.3 plastic particles/g [51–53]. Similar amounts of MP (between 0.8 and 1.7 particles per gram) were found in plant-based meals [51]. On the other hand, the method used for MP detection was different. In all previous studies, visualization of MP by a stereomicroscope and FTIR spectroscopic analysis were performed. For the identification and quantification of MP, FTIR spectroscopic analysis has limited sensitivity for MP smaller than  $10 \mu\text{m}$ , resulting in an underestimation of MP contamination.

Although a great number of MPs were detected in our insect meal sample, it is important to point out that the effects of MP on insects and other animals, such as fish, are still the subject of active research and the long-term consequences are not fully known. Potential effects include physical damage, impaired feeding and digestion, and the possible transfer of chemicals associated with plastic [18,49,54–56].

MP can indeed serve as a vector for the bioaccumulation of toxic substances in fish, and the toxicity resulting from the ingestion of plastic is a consequence of both the sorbed pollutants and the plastic material [57].

The ingestion of microplastic particles and the transfer of potentially harmful substances together with microplastics have been studied in a variety of organisms, especially invertebrates. However, the potential accumulation of very small MP along food webs ending with vertebrate models has only been investigated in a few studies so far. Results from those studies suggested that food-borne MP-associated pollutants may desorb in the fish intestine, facilitating their transfer to the intestinal epithelium and liver [57].

The regulatory context underscores the significance of addressing MP and NP in the realm of EU regulations on plastics in animal feed [58]. Recognizing the imperative to comply with these regulations, there is a pressing need for reliable identification methods specifically tailored to detect and analyse MP and NP in food. This highlights the intersection of environmental concerns, regulatory compliance, and the necessity for advanced detection methodologies in safeguarding the quality and safety of food products.

Accordingly, the application of the aforementioned methods for the detection and identification of MP and NP in insect larvae meal is a novelty in the study of this matrix. The results of KOH digestion, filter analysis, correlative SEM-Raman analysis, and superhydrophobic surface detection confirmed the presence of PE NP in the insect meal sample.

For a comprehensive analysis of MP and NP, the combination of several techniques, such as Raman spectroscopy, SEM, TEM, and EDX, can provide complementary information and overcome individual limitations [30,40].

In fact, Raman spectroscopy alone provides detailed chemical information about the composition of MP and helps to identify specific polymers. On the one hand, it is a non-destructive technique that allows the analysis of MPs without altering their structure. On the other hand, SEM provides high-resolution images of MP that allow detailed morphological analysis. SEM provides information on the surface topography and texture of microplastics and can be coupled with EDX for elemental analysis of MP. The limitations of SEM lie in sample preparation, as it often requires coating the samples, which can alter the surface properties of MP. In addition, SEM provides 2D images, which limits the ability to fully understand the three-dimensional structure of MP. While EDX can provide elemental information, SEM may not provide detailed chemical information about the polymer's composition.

TEM offers an even higher resolution than SEM and enables a detailed analysis of microplastic structures. TEM provides detailed information about the internal structure and morphology of microplastics. TEM can be combined with EDX for elemental analysis. However, as with SEM, TEM often requires complex sample preparation, and the process can produce artifacts. Working with TEM requires specialized knowledge, and the instrument is sensitive to environmental conditions. TEM has a limited field of view and is therefore less suitable for analysing larger areas.

EDX provides information on the elemental composition of microplastics and thus helps to identify them. EDX can be used for quantitative elemental analysis and provides information on the concentration of the various elements. However, the spatial resolution of EDX is lower compared to SEM and TEM, which limits its ability to provide detailed information about microplastic structures. EDX can have problems distinguishing elements from the microplastic matrix and surrounding materials. The sensitivity of EDX can be limited, especially for trace elements in microplastics.

Therefore, to thoroughly examine microplastics, utilizing multiple techniques like Raman spectroscopy, SEM, TEM, and EDX is essential. By combining these methods, which each have their own strengths and weaknesses, a comprehensive analysis can be achieved. This approach maximizes the benefits of each technique while minimizing their respective limitations.

In summary, this study provides a method to detect MP and NP in a unique matrix, offering insights into the potential presence of plastics in insect-based aquafeeds. The combination of different analytical methods strengthens the reliability of the results.

Solutions such as 'recycling' or 'circular economy' are often mentioned. Theoretically, if all used plastic was recycled and reused as new material, no more plastic would end up in the environment. Unfortunately, the reality is less rosy.

## 5. Conclusions

The main result of the current study lies in the successful discovery and identification of NP, a significant achievement in understanding the extent of plastic contamination. The identification process provides valuable insights into the presence of these minute plastic particles in the insect meal samples and potentially in insect-based aquafeeds. However, there are avenues for potential future studies that can build upon these findings.

One promising direction for future research involves the quantification of NP. While the identification of NP is a crucial first step, quantifying their concentrations allows for a more comprehensive understanding of their prevalence and potential impact. This could involve developing and refining analytical techniques to accurately measure the quantity of NP in various samples, providing data that can be used to assess the scale of contamination.

Moreover, investigating the potential effects of NP on biological systems and ecosystems represents another avenue for future exploration. Understanding the ecological implications and potential risks associated with NP exposure can contribute to the development of informed regulations and mitigation strategies.

Additionally, exploring the sources and pathways of NP pollution can be a crucial aspect of future studies. This could involve tracing the origins of NP in different environments, understanding how they enter food chains, and identifying potential sources for effective pollution prevention measures.

In summary, the current study successfully identifies and characterizes NP in insect meal. This procedure is often complicated and unique for each matrix. Therefore, there is room for future research to focus on quantification methods, ecological impacts, and the sources of NP pollution. These attempts will contribute to a more comprehensive understanding of the issue and aid in the development of strategies to mitigate the environmental impact of NP contamination.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/environments11060112/s1>, File S1.

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**Data Availability Statement:** All the data supporting the reported results can be found in this article.

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