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RESEARCH ARTICLE

Determination of microplastics in reared black soldier fly larvae (*Hermetia illucens*) using polarised light optical microscopy

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Abstract

Microplastics have become ubiquitous in the environment and are increasingly found in a variety of matrices, including in animal feed. At the same time, the presence of plastics from packaging materials in animal feed is still prohibited in the European Union. This is a major barrier preventing certain organic waste streams from being repurposed as feed. However, issues associated with the presence of plastic particles in feed for 'conventional' livestock such as poultry and pigs (e.g. accumulation in stomach/other tissues) may not pose a problem to reared insects such as black soldier fly larvae (BSFL). In the hypothetical absence of such transfer, insects reared on feed contaminated with plastics could potentially be repurposed as feed. Such reuse would be a major opportunity for a more circular food chain. The aim of this exploratory study was to develop a method to determine the potential transfer of microplastics from the substrate to biomass of reared BSFL. Tested plastics consisted of low-density polyethylene (LDPE) of three different sizes, and polyethylene terephthalate (PET); to mimic the types of synthetic polymers commonly used for food packaging. The results showed no significant effects of dietary exposure of tested (micro-)plastics on larval yield and survival. We benchmarked fluorescence microscopy through Nile Red staining against a newly developed technique utilizing polarised light optical microscopy to identify plastic particles in BSFL. Tested LDPE particles were virtually absent from the post-exposure larvae. PET was recovered from the larvae, although it is unclear what the exact mechanism for this transfer was. More research is needed to validate these experimental findings with plastics of different types and particle sizes.

Keywords

BSFL – circularity – packaging

1 Introduction

Insects are seen as a potential novel food and feed source (Barragan-Fonseca *et al.*, 2017; Bosch *et al.*, 2019; Wang and Shelomi, 2017). In particular, polyphagous insects such as the black soldier fly larvae (BSFL, *Hermetia illucens* (L.); Diptera: Stratiomyidae) are able to valorise a wide variety of non-edible by-products, which would otherwise be lost from the food chain (Bava *et al.*, 2019; Liu *et al.*, 2019; Salomone *et al.*, 2017). These insects are therefore expected to play a major role in the protein transition and circular economy (Bortolini *et al.*, 2020; Ojha *et al.*, 2020). One such type of waste materials is former foodstuffs (FFS), described in EU

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legislation as foodstuffs that due to practical or manufacturing defects are no longer intended for human consumption (Regulation (EU) No 68/2013). One issue with the processing of FFS into feed is the separation of packaging materials from the biomass (Amato *et al.*, 2017; Calvini *et al.*, 2020; Van Raamsdonk *et al.*, 2011). In the EU, it is completely prohibited for food packaging materials to be present in feed (Regulation (EC) No 767/2009, Article 6 and Annex III). The presence of packaging materials in feed would make it unsuitable for conventional livestock such as pigs and poultry, since they may consume these plastics but are unable to digest them, and the smallest particles may even pass gut barriers into other organs (Bové e*t al.*, 2019; Leslie *et al.*, 2022; Ramachandraiah *et al.*, 2022; Van-der Veen *et al.*, 2022).

Besides chemical risks (Van der Fels-Klerx *et al.*, 2020), there is the probability of accumulation or transfer of microplastics, or degradation of larger particles into microplastics (MPs; defined as particles between 1 and 5,000 μm; Van Raamsdonk *et al.*, 2020). MP particles have increasingly been found in the environment, particularly marine ecosystems (Petersen and Hubbart, 2021; Sarijan *et al.*, 2021; Shahul Hamid *et al.*, 2018; Vitali *et al.*, 2023; Wang *et al.*, 2022), and human exposure has risen concurrently (Kannan and Vimalkumar, 2021; Zhang *et al.*, 2020). Reviews by EFSA (2016) and van Raamsdonk *et al.* (2020) concluded that no risk assessment on the presence of microplastics could be performed due to lack of data – but both studies remarked that this lack of evidence points to a data gap, and not the absence of risk (EFSA, 2016; Van Raamsdonk *et al.*, 2020). This lack of data is pointed out in more recent publications as well (Koelmans *et al.*, 2022; Thornton Hampton *et al.*, 2022). Globally, the most used type of plastic in packaging is polyethylene (PE): low-density polyethylene (LDPE) and linear LDPE (LLDPE) accounted for 30% of use in 2009, followed by high-density polyethylene (HDPE) at 26% and Polyethylene terephthalate at 18% (PET). PE food packaging is used for, *inter alia*, "bakery, meat, poultry and dairy products, [and] frozen food packaging" (Katiyar *et al.*, 2014). A variety of detection, identification, and quantification methods have been described in literature (overview in Van Raamsdonk *et al.*, 2020): optical vibrational spectroscopy (micro-FTIR and micro-Raman spectroscopy), thermal analysis (e.g. pyrolysis gas chromatography-mass spectrometry), fluorescent tagging with Nile Red (Shruti *et al.*, 2022; Stanton *et al.*, 2019; Vitali *et al.*, 2022), Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) (Toussaint *et al.*, 2019), and polarised light optical microscopy (Sierra *et al.*, 2020). Each of these methods has advantages and disadvantages, which make them more or less suitable depending on the matrix and objective.

MPs have been found in a variety of insects (Goveas *et al.*, 2023; Oliveira *et al.*, 2019). Certain mandibulate insect species have long shown a capacity to degrade synthetic polymers, such as the larvae of lesser mealworm (*Alphitobius diaperinus* (Panzer); Coleoptera: Tenebrionidae) (Ichinose *et al.*, 1980; Vaughan *et al.*, 1984). Therefore, several studies have been published focusing on the effects of MPs in insects. For instance, Romano and Fischer (2021) found that polypropylene (PP) microplastics in diet negatively affected pupation of BSFL. Furthermore, Lievens *et al.* (2023) exposed BSFL to dietary fluorescent blue-labelled microplastics with a median size of 61.5 μm and dissected larvae of different ages to isolate the gut contents. Using stereo and optical microscopy, they concluded that tested microplastics were present in the gut of exposed BSFL, but that ingestion would depend on larval mouth opening dimensions (and thus age).

Research on the potential of rearing insects on FFS containing packaging materials is required to close the currently existing research gaps. We hypothesise that the presence of MPs may not pose a considerable hazard to reared insects such as BSFL, due to their comparatively smaller size. Therefore, the goals of this exploratory research were (1) to identify a suitable and cost-efficient optical detection method for detection of MPs (detection microplastics), (2) to develop a digestion method to remove organic material (i.e. the larval matrix) from the MPs without affecting the integrity of the tested plastics (matrix destruction), (3) putting the complete method to the test by rearing BSFL on substrate spiked with PET and LDPE (detection of MPs in experimental samples), and (4) determining the effect of the MP presence on the larvae (survival and yield). Achieving these goals results in an indication of the potential of BFSL to degrade, accumulate, or otherwise transfer microplastics when reared on substrates containing LDPE or PET.

2 Materials and methods

Experimental design

An overview of the total study design with the experiments and supporting method development is presented in Figure 1. Two studies were performed. In the first study, larvae were exposed to 0.5 g (1%), 1.5 g (3%),

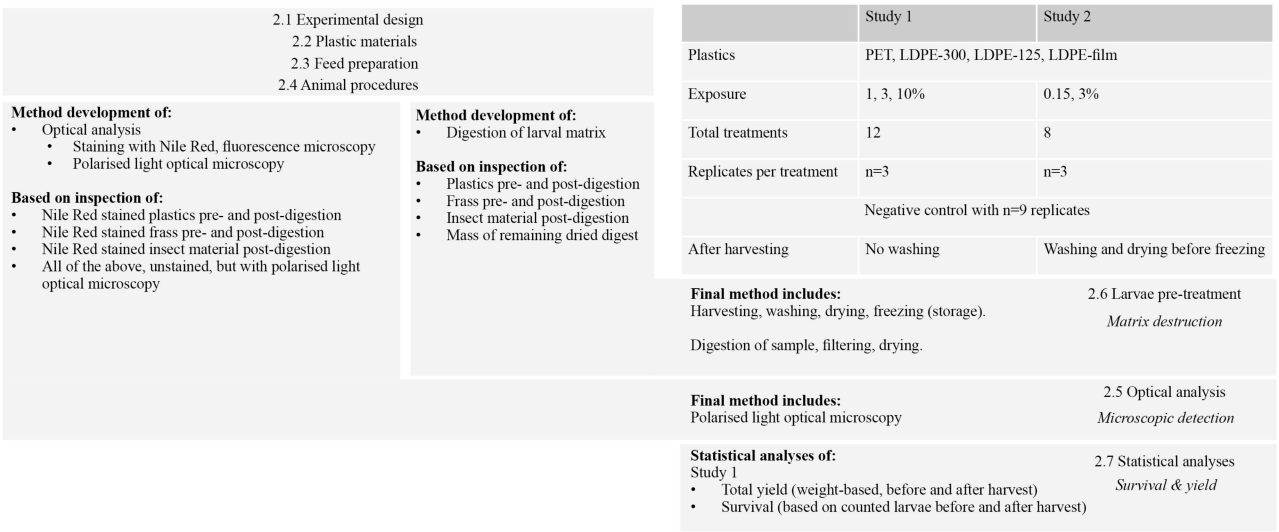

FIGURE 1 Graphic summary of the experimental design, following the applied work-flow from top to bottom and the logical order of method development from left to right, with references to corresponding subsections in materials and methods.

and 5 g (10%) of plastics present in the diet. With four different types of plastic materials, this results in a total of 12 different treatments. In the second study, the exposure was 0.075 g (0.15%) and 1.5 g (3%). With four different types of plastic materials, this results in a total of 8 treatments. A diet without any additional plastic was used as the negative control in each study. The treatments were performed with n = 3 replicates each and the negative control was conducted with n = 9 replicates. This results in a total of 45 samples for the first study and 33 for the second. An overview of the experimental design and number of replicates is depicted in Table 1.

The concentrations in study 1 (1, 3, and 10%) represent levels above the Dutch Reference point of Action of 0.15% for presence of packaging materials in former food products (Van Raamsdonk *et al.*, 2011). In study 2, exposure of 0.15% was included to reflect the Reference point of Action, as well as a higher concentration of 3%.

Plastic materials

Four different types of plastic materials were used in this study, being either polyethylene terephthalate (PET) or low density polyethylene (LDPE) in various sizes as shown in Table 2. These types of plastic were selected, because they are most commonly used as packaging materials for food. The tested synthetic polymers consisted of two commercially available powdered MPs (PET and LDPE), LDPE microspheres, and an LDPE film that originated from standard transparent LDPE bags intended for loaves of bread (thickness: 20 μm; dimensions: 18 × 4 × 50 cm) (Brabo Verpakking, Breda, the Netherlands), cut up into pieces of approximately 5 × 5 mm each. This latter material was selected to simulate the size of films of packaging materials that were expected to be common in FFS – if that practice were to be permitted. Different types of morphology are considered by including film, microspheres and powders. PET and LDPE powder of 300 μm allowed direct comparison based on plastic type, because the morphology and size are the same. Finally, LDPE microspheres of 125-150 μm were the smallest size to be included.

Feed preparation

The replicate containers were standard petri-dish-like insect rearing containers (diameter 100 mm, height 40 mm) with a mesh built in the lid for ventilation (SPL Life Sciences Co., Ltd., Gyeonggi-do, South Korea). To ensure the larvae were presented with sufficient and equal amounts of feed, the amount of plastic materials was added as specified per treatment to 50 g of substrate per replicate container, and subsequently mixed until all components were evenly distributed. By applying this spiking procedure, the amount of nutrients is the same for each replicate, assuming that the plastics are inert and do not provide any nutritional value to the larvae, if ingested. The substrate consisted of 29% maize-based compound feed (pellets for laying hens (Kasper Faunafood, Woerden, the Netherlands)), 10% horse bran (Horsefood, Waalwijk, the Netherlands), and 61% tap water.

Animal procedures

After distributing the substrate over the replicate containers and performing the spiking procedures at the analytical laboratory (Wageningen Food Safety Research

TABLE 1 Overview of the experimental design indicating the treatments, conditions and replicates for each study

Treatments	Plastic type	Exposure (%)	Replicates (n)
Study 1			
1	PET	1	3
2	PET	3	3
3	PET	10	3
4	LDPE-300	1	3
5	LDPE-300	3	3
6	LDPE-300	10	3
7	LDPE-125	1	3
8	LDPE-125	3	3
9	LDPE-125	10	3
10	LDPE-film	1	3
11	LDPE-film	3	3
12	LDPE-film	10	3
	Negative control	0	9
			Total = 45
Study 2			
1	PET	0.15	3
2	PET	3	3
3	LDPE-300	0.15	3
4	LDPE-300	3	3
5	LDPE-125	0.15	3
6	LDPE-125	3	3
7	LDPE-film	0.15	3
8	LDPE-film	3	3
	Negative control	0	9
			Total = 33

TABLE 2 Overview of materials used in treatments with plastics, specifying size and supplier

Material type (further referred to as)	Size	Supplier
Polyethylene terephthalate (PET) powder (PET)	300 μm	Goodfellow GmbH (Hamburg, Germany)
Low density polyethylene (LDPE) powder (LDPE-300)	300 μm	Goodfellow GmbH (Hamburg, Germany)
LDPE microspheres (LDPE-125)	125-150 μm	Cospheric (Santa Barbara, CA, USA)
Shredded LDPE bags (LDPE-film)	~5 mm	Brabo Verpakking, Breda, the Netherlands

(WFSR), Wageningen the Netherlands), the rearing containers with the substrate were transferred to the insect rearing facilities at Protix B.V. (Dongen, the Netherlands) for the subsequent controlled feeding trial. Per replicate, seven-day-old larvae (n = 100) were weighed and placed in each rearing container. The weights of the larvae per treatment and per replicate were stored for statistical analysis. The larvae were reared for seven days. The rearing containers were kept in climate cabinets (ICH 110, Memmert, Germany, d = 0.1 °C) at 32 °C and 70% relative humidity. After 7 days, the larvae from the replicate containers of each treatment were removed from the container, counted, and weighed. The remaining substrate after rearing, i.e. the frass, was not discarded but stored at –18 °C in its original container for further examinations. In study 2, the larvae were also washed and dried. Washing was done in a continuous conveyor system in which live larvae were suspended in water. A full description of this system is provided in patent number WO2020246879A1. In summary, the larvae were transferred into a water bath at 10-20 °C and suspended for 6 hours with a water-larvae mass ratio of 1:3. This step was intended to clean the surface area of the larvae, as well as flushing their gut contents. After drying with paper towels, the larvae were frozen at –18 °C as a kill-step and held frozen. The frozen samples

were subsequently transferred back to WFSR for analysis.

Optical analysis (*microplastic detection*)

Two different approaches for optical analysis (i.e. staining with Nile Red and polarised light optical microscopy) were considered. Slides were prepared using the dried, digested materials. For the Nile Red stained sample slides, staining was performed by making a Nile Red (Sigma-Aldrich, technical grade, Steinheim, Germany) stock solution of 50 mg/L in acetone (Ar grade, Actu-All Chemicals, Oss, the Netherlands). This stock solution was then diluted in n-hexane (PEC grade, Actu-All Chemicals, Oss, the Netherlands) to a concentration of 10 μg/L. A small amount of the dried filter residue was added to a 1.5 mL Eppendorf tube, to which 250 μL of dye solution was added, followed by 0.75 mL of demineralised water. The tube was then shaken manually and left to stand for at least 15 minutes in darkness. Samples were transferred onto a glass microscope slide (plain slides 25 × 75 × 1 mm, Thermo Fisher Scientific, Waltham, MA, USA) using a spatula and left to air dry in darkness. The samples were surrounded by a layer of parafilm onto which a glass coverslip was placed, in order to prevent sample contamination and loss. Next, these samples were visually inspected with fluorescence microscopy using an Olympus BX51 microscope equipped with a WIBA filter (excitation 460-490 nm, emission 510-550). Images were taken with an attached Olympus SC50 CMOS camera (Olympus Corporation, Tokyo, Japan).

For polarised light optical microscopy, slides were prepared by placing a small amount of sample on a glass microscope slide. Three drops of paraffin oil (Sigma-Aldrich, Steinheim, Germany) were placed on the sample using a dropper for use as an embedding agent, followed by a glass coverslip. Next, these samples were visually inspected and imaged with and without a polarising light filter using an Olympus BX51 microscope with an Olympus U-ANT polarising filter, an Olympus UTP530 Wave Plate, and an attached Olympus SC180 CMOS camera. Images were taken at 100× and 200× magnification. All images were acquired using the software cellSense (Olympus Corporation, Tokyo, Japan).

Larvae pre-treatment (*matrix destruction*)

In order to isolate the microplastics from the organic material of the larval matrix, a pilot study was set up based on methods described in literature available at the time (see Appendix A) to determine the optimal sample pre-treatment to isolate the microplastic material from the organic material of the larval matrix or the frass. To determine the effect of the digestion method during development, pre- and post-digestion observations were performed of the insects, frass, and microplastic materials. These observations consist of images of control samples, including samples of the selected plastics both pre- and post-digestion as positive controls, and pre- and post-digestion frass and post-digestion insect materials as negative controls. Furthermore, the weight of the dried insect digest on the filters was determined to estimate the achieved loss of the matrix, as well as the pre- and post-digestion weight of the selected plastics. As such, 100 mg ± 1 mg of plastic was weighed out and digested according to the method. Before filtration, the filter paper used was weighed as well, and after filtration and drying, the filter plus microplastics were weighed in order to determine any loss of plastic by the digestion method. The final optimised sample preparation method was as follows. After receiving the frozen samples at WFSR, the larvae were washed by putting them in a sieve and rinsing with lukewarm tap water. After thoroughly rinsing, the larvae were dried carefully using a paper towel. Then, larvae (and separately, the frass) were coarsely ground using a porcelain mortar and pestle to produce particles in the appropriate size for microscopic examination. The mortar and pestle were cleaned after every sample using running tap water and a dish brush. Next, a digestion was applied to dissolve most of the matrix. All chemicals used in this procedure were supplied by Merck KGaA (Darmstadt, Germany). For every sample, 1.0 ± 0.2 g of the material was added to a plastic Greiner tube, to which a 10 ml mixture of 0.07 M diluted hydrochloric acid (HCl, suprapure, 30%) and 3% (v/v) hydrogen peroxide (suprapure, 30%) was added. The sample was digested in a water bath (Julabo TWB-12; Julabo Nederland B.V., Boven-Leeuwen, The Netherlands) for 4 hours at 80 °C. The digest was cooled to room temperature and filtered over a weighed cellulose filter with 2.5 micrometre pores (Whatman plc, Maidstone, UK). The filter was air-dried at room temperature (~20 °C) for at least one day in a drying cabinet. The dried filter was weighed again to determine the weight of the residue on the filter, after which it was prepared for optical analysis.

Statistical analyses (*survival and yield*)

The total yield of the larvae was determined by weighing all harvested larvae from each replicate. Then, the average was calculated based on the three replicates for each treatment, or based on nine replicates in case of the negative control, with corresponding standard deviation.

For larvae survival, living larvae were counted for each replicate during harvesting. Then, the average number of larvae was calculated for each treatment with corresponding standard deviation. The average number was then divided and multiplied by 100, relating to the original number of 100 larvae that were used for each replicate. This results in the larval survival in percent.

Statistical differences for the variables survival and total yield (study 1, based on counted larvae before and after harvesting and the weight of the larvae before and after harvesting) were tested for significance by using non-parametric tests (Kruskal-Wallis, $\alpha = 0.05$). Statistical tests were performed using the software SPSS Statistics for Microsoft Windows (version 25.0.0.2, IBM Corp., Armonk, NY, USA).

3 Results

Method development: microscopic detection

To determine the best microscopic detection method, samples were inspected after staining with Nile Red followed by fluorescence microscopy, and unstained with bright field using a polarising filter. Inspected samples (further referred to as the control samples) included samples of the selected plastics both pre- and post-digestion (positive controls), pre- and post-digestion frass, and post-digestion insect material (the latter all being negative controls). Fluorescence microscopy showed that Nile Red stained both positive and negative controls (Figure 2). Furthermore, visual inspection showed that the residue mainly consisted of larvae cuticle and microplastics, if present in the sample.

When observing unstained control samples with polarised light optical microscopy, it was found that both PET and LDPE-300 show a highly distinctive iridescent coloration pattern (Figure 3). However, LDPE-125 only shows an iridescent coloration pattern at the edges of the particles and not on the surface itself. The pattern was dependent on particle shape and size, with the generally flat LDPE-film showing a smooth coloration and the more irregularly shaped microplastic particles showing an irregular, iridescent, coloration. The frass, both pre- and post-digestion, consisted mostly of plant material. Plant material generally shows a more or less faint colorization pattern during polarised light optical microscopy too, but their cellular and/or fibrous structures make them easily identifiable, and thus distinguishable, from plastic particles. The digested insect material showed little to no polarisation, with the cuticle fragments generally staying clear and transparent.

Approximately 1% of cuticle fragments showed polarised coloration within their structure (Figure 4). The colorization patterns remained after embedding in demineralised water and showed no reaction to embedding in a silver nitrate solution, which made it unlikely that the coloration was due to a possible deposition of salts as a remnant of the digestion process. In some cases, certain appendages present on the exoskeleton of the larvae could be found in the sample, and these showed a clear and mostly smooth colorization, rather than the iridescent pattern observed in the polymers. The morphology appears to be consistent with palps, described by Oliveira *et al.* (2016). Due to the different colorization patterns, plastics were still readily identifiable and distinguishable from insect particles.

Based on the observations of Nile Red co-staining the matrix and polarisation allowing easier identification of microplastic materials in the residue, it was decided that optical detection under bright field with a polarising filter was preferred over staining with Nile Red followed by fluorescence microscopy.

Method development: matrix destruction

The sample pre-treatment aimed to remove as much matrix material as possible, while leaving a residue of unaltered microplastics on the filter. Based on methods available at the time in literature (see Appendix A), different chemicals were tested, such as sodium hydroxide, hydrogen peroxide after sodium hydroxide, and hydrochloric acid combined with hydrogen peroxide, as well as different digestion times and temperatures. When digesting 1 g of insect material with the optimal digestion method, 11.0 ± 0.7% of the original mass was found as residue on the filter. Microscopic investigation, using both Nile Red staining and polarised light microscopy, did not show a noticeable difference between pre- and post-digestion samples.

Detection of microplastics in experimental samples

After testing the developed sample pre-treatment and detection methods on the samples from study 1, it was established that leaving the larvae unwashed after harvesting could result in plastic material or frass sticking to the larval exterior. Contamination of plastic particles from the frass could thus not be excluded, making the results for presence of microplastics in study 1 only valuable for method optimization. Therefore, the larvae were washed after harvesting to eliminate the risk of contamination from the frass in the second study, besides limiting the concentrations to 0.15 and 3%. Due to the eliminated risk of contamination regarding the

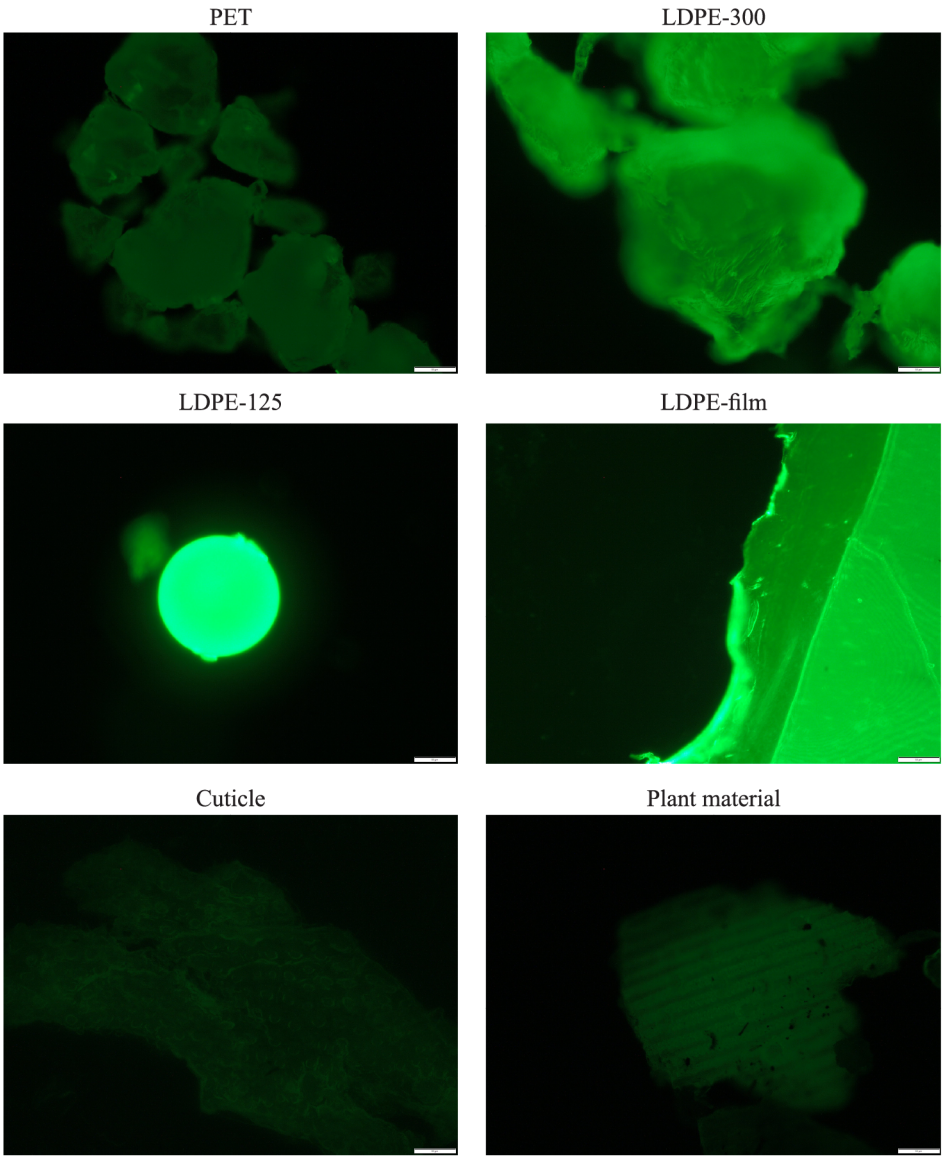

FIGURE 2 Representative images of the Nile Red stained control samples post-digestion. Both the positive plastic control samples (PET, LDPE-300, LDPE-125, and LDPE-film) and the negative control samples (insect cuticle/particles and frass) show staining by Nile red. The depicted plant material was a part of the negative frass control. Scale bars represent 50 μm.

harvesting process, the presence of microplastics will only be discussed for study 2.

Plant material was found only in trace amounts in all digested insect samples (i.e. <1% of particles (irrespective of size), and all but one treatment consist mostly of cuticle particles. At the 0.15% spike level, samples of LDPE-300, LDPE-125, and LDPE-film treatments did not show any plastic particles, while at 3.0% spike level the LDPE-300, LDPE-125, and LDPE-film treatments showed only trace amounts, i.e. <1% of particles, of plastic particles. Both spiked levels of PET, on the contrary, showed considerable amounts of plastic particles, with the 0.15% spike level containing ±10% plastic particles of all particles present, and the 3.0% spike level containing >50% plastic particles of all particles present. The results are summarised in Table 3. Furthermore, representative images of the different types of plastics found in insect samples can be found in Figure 5.

Larval survival and yield

Determining the larval survival and yield could give an indication of the economic feasibility of rearing BSFL on plastic-containing substrates. Since three different spike levels were used in study 1, including a high level of 10%, those results were used to calculate the larval survival and yield. Based on the weight and counted number of larvae before and after harvesting of study 1, the total yield and survival were calculated, respectively.

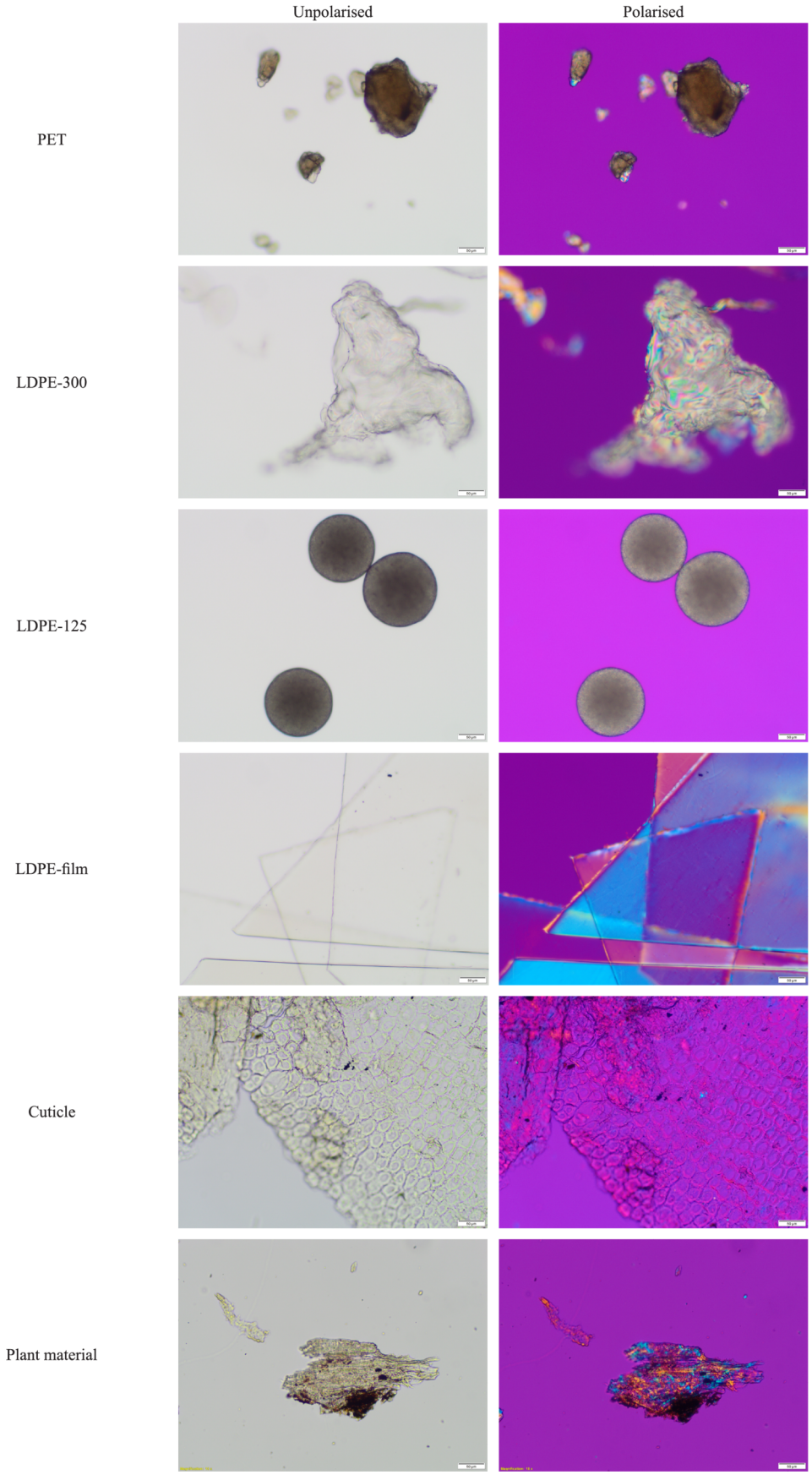

FIGURE 3 Representative images of the unstained control samples post-digestion, both with and without polarisation filter. Scale bars represent 50 μm.

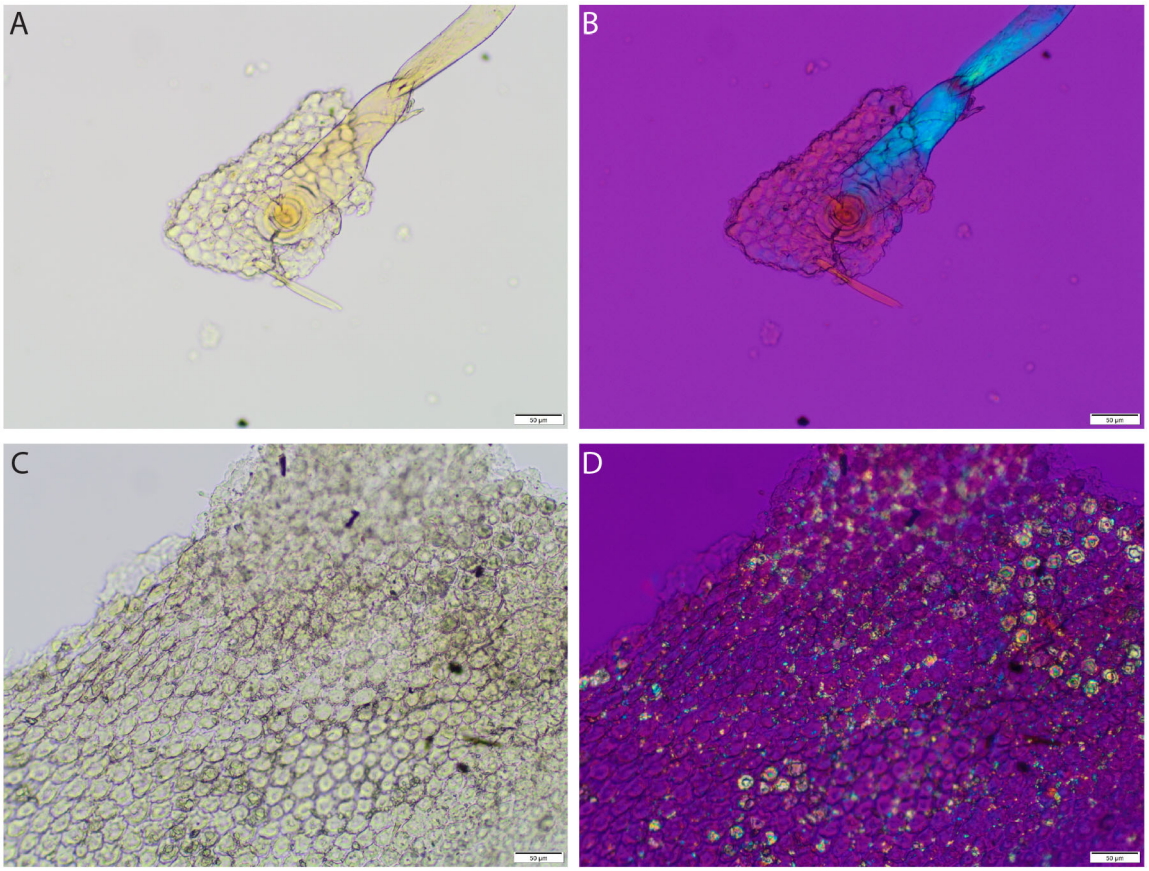

FIGURE 4 Representative images of insect particles post-digestion that show an iridescent polarisation pattern. (A-B) Unpolarised (A) and polarised (B) images of an appendage, and (C-D) unpolarised (C) and polarised (D) images of a rare (<1%) insect particle that shows an iridescent polarisation pattern. Scale bars represent 50 μm.

TABLE 3 Summary of the results of study 2, regarding the presence of microplastics in eight different treatments by visual inspection with polarised microscopy

Plastic material	Spike level	Presence of microplastics in insect samples
PET	0.15 %	± 10 % of all particles, plastic
LDPE-300	0.15 %	Not present
LDPE-125	0.15 %	Not present
LDPE-film	0.15 %	Not present
PET	3.0 %	> 50 % of all particles, plastic
LDPE-300	3.0 %	Trace amounts present
LDPE-125	3.0 %	Trace amounts present
LDPE-film	3.0 %	Trace amounts present

Differences between treatments in terms of total yield (P = 0.269) and survival (P = 0.707) were not significant. The results per treatment are shown in Table 4.

4 Discussion

This exploratory study focused on four different goals, of which the first focused on microplastic detection by identifying a fast and cheap optical detection method. Two methods of optical analysis were compared. Optical detection with microscopy was chosen, because it is rapid and cheap compared to other methods such as FTIR. As a benchmark, samples were stained with Nile Red and imaged using fluorescence microscopy, as this appears to be the most commonly used method for similar types of matrices at the moment (Shruti et al., 2022; Stanton *et al.*, 2019; Vitali *et al.*, 2022). An additional

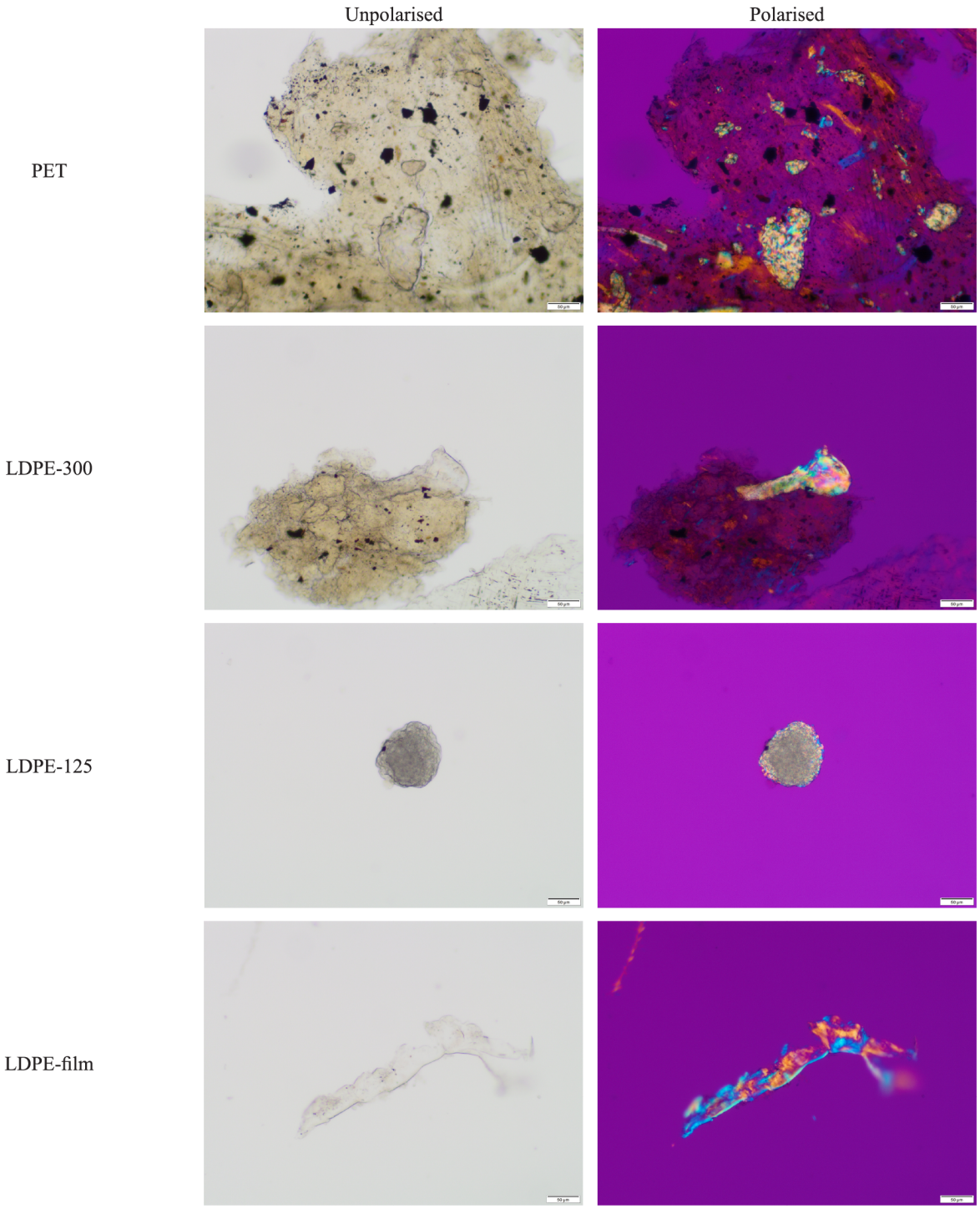

FIGURE 5 Representative unpolarised and polarised images of plastics found in digested insect samples from study 2. Scale bars represent 50 μm.

method to identify the plastic particles using polarised light optical microscopy was attempted, because this was considered to be a faster and cheaper alternative. We found that Nile Red did indeed reliably stain the plastic particles. However, the negative controls (pre- and post-digestion frass, and post-digestion insect material) showed that Nile Red also stained the cuticle particles of the pure insect material and the plant material in the frass. Therefore, the method employing Nile Red was concluded to be suboptimal as a way to identify and distinguish microplastic material from the insect cuticle – especially in the potential case of microplastic adhering to, or somehow being incorporated into the cuticle. When studying the positive and negative controls under

TABLE 4 Total yield and survival (mean and standard deviation), calculated based on the weight and counted number of larvae before and after harvesting of study 1. For the spiked treatments, 3 replicates were included, while for the negative control 9 replicates were included

Type of material (size)	Percentage plastics added to feed (%)	Total yield (g)	Survival (%)
PET	1	10.9 ± 0.5	99.3 ± 0.6
	3	11.1 ± 0.2	99.7 ± 0.6
	10	10.9 ± 0.3	99.3 ± 0.6
LDPE-300	1	11.2 ± 0.3	100.3 ± 0.6
	3	11.5 ± 0.2	99.7 ± 0.6
	10	10.8 ± 0.3	100.0 ± 0.0
LDPE-125	1	11.0 ± 0.4	99.3 ± 1.2
	3	11.3 ± 0.4	100.3 ± 1.2
	10	10.9 ± 0.7	99.0 ± 1.0
LDPE-film	1	11.2 ± 0.1	99.3 ± 1.2
	3	12.2 ± 1.1	99.3 ± 0.6
	10	11.4 ± 0.2	99.7 ± 1.2
Control	n/a	11.2 ± 0.5	99.7 ± 0.7

polarising light microscopy, we found that plastic, cuticle, and plant materials all had strongly distinguishing features, which allowed for the rapid identification of particle origin. It is important to emphasize that simply the presence of a polarising pattern is insufficient to distinguish MPs from other particles such as cuticles or plant materials. One of the most important criteria for identifying MPs is the absence of, for instance, cellular structures. Therefore, the knowledge of a well-trained microscopist is necessary, being able to recognize different types of particles and assigning their identity correctly. Using polarisation, no staining was necessary, and the particles could be distinguished even better than with Nile Red staining. As such, we opted to continue with polarised light optical microscopy as the method of detection during this exploratory study.

The second goal involved matrix destruction. To be able to study the microplastic particles microscopically, removal of the matrix was required to reduce or minimise interference of the matrix for microscopic detection. While sample pre-treatment for chemical analyses generally entails extraction of the target substance, the focus of sample pre-treatment for microscopic analysis of microplastics involves "primarily the destruction and/or removal of the matrix material" (Van Raamsdonk *et al.*, 2020). Isolating MPs from liquid matrices generally only requires sieving, and potentially adding reagents such as H_2O_2 to separate MPs from remaining organic material (Raj and Maiti, 2023). However, analysis of MPs in more complex solid matrices (such as insects) requires more intensive methods to remove all organic material without affecting the integrity of the tested plastics, which necessitates a delicate balance (Monteiro and da Costa, 2022; Ruggero *et al.*, 2020). In order to accomplish this, the samples were digested in 0.07 M HCl and 3% (v/v) hydrogen peroxide. To determine the effects of the digestion method, its effects on the matrix and MPs were studied. The final optimised digestion method was successful in removing the vast majority of insect material which consists primarily of proteins. Microscopic investigation showed no noticeable differences between MPs pre- and post-digestion. Furthermore, no difference was observed in the staining efficiency with Nile Red, nor in the polarisation patterns observed in the microplastics post-digestion. This suggests that no major chemical alterations have taken place. Hence, the developed digestion method sufficiently concentrated the microplastic particles without affecting them, allowing the samples to be microscopically analysed. However, this method did not succeed in completely digesting away the insect cuticles, which (mostly) consist of chitin. Use of chitinase enzymes to break down this remaining organic matter was briefly considered as an additional step in digestion: however, since the tested plastics were easily identifiable and the microplastics appeared to be unaffected by the digestion, the currently developed digestion method was regarded fit for purpose. What's more, the presence of cuticle particles in the residue could possibly lead towards semi-quantification, if the number of particles in the residue would be comparable across different samples. The presence of the cuticle particles could then be used as a pseudo-quantitative internal standard, by comparing the number of plastic particles to

the number of cuticle particles in the sample. Exploring such a semi-quantitative method could be considered for future research, which could assist in filling the currently existing research gaps. Nevertheless, it is anticipated that the existing 'zero-tolerance' for plastics in animal feed for conventional livestock (pigs, poultry, etc.) will remain in place. Therefore, any insects reared on substrates containing plastics should be free of any transferred particles regardless: qualification of MPs in insect materials intended as feed can therefore be considered sufficient for monitoring purposes. Finally, the lack of plastic particles found in the negative controls showed that there was no issue with environmental contamination of microplastics.

Putting the entire method to the test meant achieving the third goal of this study. Detection of MPs in BSFL was performed on four different treatments at two spike levels (study 2, section 3.3). When looking at BSFL reared on the LDPE-300, LDPE-125, and LDPE-film spiked substrates, no plastic was found in the digested samples at the low spike level and only trace amounts of plastic were found at the high spike level. This could imply that insects reared on feed spiked with LDPE hypothetically either avoid ingesting the plastic, or degrade and/or excrete it, or some combination of this. A relatively high level of plastic particles in the PET-spiked samples was found, which could indicate that the BSFL did not actively avoid the PET particles in the feed and that some form of transfer by the BSFL could have taken place. Since particle sizes of spiked PET particles were in the same size range as the LDPE-300 particles, it could imply that size was not a strongly inhibiting factor in this case. This is contrary to what has been described in literature (Lievens *et al.*, 2023), where ingestion of MPs by BSFL was suggested to depend on particle size, the concentration present in the substrate and the larval age or size. Other possible relevant factors could include the chemical structure of the polymer (PET vs LDPE), the density of the particles and the shape, making the transfer of MPs to BSFL a multifactorial process. Therefore, it is recommended to take these factors in account in future research, which was also recommended by Lievens *et al.* (2023). Another aspect to be considered is the possibility that the plastic particles found were not ingested by the BSFL, but that they merely clung to the exterior of the larvae. Whilst this is theoretically possible – as we would have no way of differentiating this with the used sample preparation method – it is highly unlikely, as the insects were thoroughly washed and visually inspected after washing for remaining materials. If the washing was insufficient, it is likely that ample plant material in these samples would also have been observed, which was not the case. It is believed that washing the larvae for six hours empties their gut contents, but this must be validated. There are concerns on the effects of extended periods of water immersion on insect welfare, but this should be studied further (Barrett *et al.*, 2023).

Finally, to obtain an indication of the economic feasibility of rearing BSFL on plastic-containing substrates, the survival and total yield of the BSFL were determined (study 1, section 3.4). The survival and total yield of the BSFL were not significantly affected, which was in line with earlier reported results that BSFL are not affected by the presence of PE or PET in the feed (Van der Fels-Klerx *et al.*, 2020). Any adverse sub-lethal effects resulting from MP exposure, such as decreased pupation rates as found by Cho *et al.* (2020) and Romano and Fischer (2021), were out of scope but require further investigation.

5 Conclusion and recommendations

This exploratory study suggests that transfer of microplastic particles to BSFL via dietary exposure is negligible for one of the most common plastic types, namely LDPE. Further research is recommended to collect sufficient data to fill the currently existing research gaps. If further research shows that this does indeed lead to the minimal transfer of plastic materials in insect feed to the BSFL, a risk assessment based on these data could be performed, possibly leading to potentially rearing BSFL on plastic contaminated feeds. This would be a considerable gain to utilizing BSFL in more circular livestock management systems and to reduce the ecological footprint associated with the production of feed and foodstuffs. However, additional studies must first confirm that the observed lack of microplastic transfer is also the case for a larger variety of types, particle sizes, shapes and densities. In particular, more research is needed on the exact transfer mechanism which resulted in the observation of PET in larval samples. Finally, it is recommended to further investigate the effect of washing the larvae for an extended period of time, which could potentially allow them to empty their guts, thus affecting the presence of MPs.

The main take-away of this study is intended to be that HCl+H_2O_2 digestion followed by polarised light optical microscopy is a simple, relatively high-throughput method of analyzing LDPE and PET (micro)plastic particles in the ranges of 1 μm – 5 mm. We

believe this method shows great promise in the field of microplastic detection. It would be worthwhile testing the developed digestion and analysis methods on reared insect species that have shown a capacity to degrade and ingest synthetic polymers, such as *Alphitobius diaperinus* and *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae). Finally, it is recommended that the developed analytical method is benchmarked against other methods for microplastic detection.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.27602283

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Author contributions

NLD contributed to methodology, writing – review & editing. GvdB contributed to methodology, formal analysis, data curation, writing – original draft, visualization. AH contributed to conceptualization, design and execution of the experiments, writing, and project coordination. LWDvR contributed to conceptualization, methodology, supervision, writing – review. RZ contributed to the execution of the experiments and project coordination. ES contributed to conceptualization. BH contributed to methodology, investigation. SR contributed to methodology, investigation. GvB contributed to methodology. NM contributed to conceptualization, methodology, writing – review & editing, project administration, funding acquisition.

Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics

This study complies with relevant institutional, national, and international guidelines and legislation. No endangered species, or at risk of extinction, have been used for this research.

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