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UPTAKE AND EGESTION OF POLYHYDROXYALKANOATE MICROBEADS IN THE MARINE COPEPOD *Nitokra lacustris pacifica*

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Abstract: Petrochemical-based microplastics are widely distributed and abundant in the marine environment, and they have become a global economic and environmental concern. Microplastics are mistakenly ingested by zooplankton as food, subsequently disrupting the biological process of zooplankton, a crucial food source for many secondary consumers. Research has led to the development of biodegradable plastics, such as polyhydroxyalkanoate (PHA), to remediate this problem. This study aims to evaluate the ingestion and egestion of PHA microbeads by the *Nitokra lacustris pacifica* copepod. The polymer P(3HB) produced from *Massilia haematophila* UMTKB-2 was used to develop PHA microbeads. The PHA microbeads were then added to copepod cultures to study the ingestion of the PHA microbeads and its effects on the copepods. The copepods were viewed under an inverted fluorescence microscope to confirm the presence of the PHA microbeads in their digestive tracts and faecal pellets. The percentage of PHA microbeads ingested by the copepods and their incorporation in faecal pellets were higher, > 70% and > 80%, respectively, after being exposed for 24 h and three days were required to fully egest the microbeads. However, no mortality was observed in this short-term incubation experiment.

Keywords: Polyhydroxyalkanoate, microplastics, zooplankton

Introduction

The application and discharge of plastics or plastic-containing waste pose a threat to wildlife and pollute the environment as they decompose very slowly, eventually accumulating in the ultimate sink for plastic waste - the ocean. Petrochemical-based plastics have been widely used in numerous consumer products for daily use, as well as for household, packaging, cosmetic, medical and agricultural purposes. The forms of petrochemical-based plastics include microplastics found in most commercial products for personal hygiene and domestic cleaning, such as toothpastes, scrubs, shampoos, soaps, facial cleansers, and abrasive cleaning agents. Microplastics are among the chief plastic waste in the ocean, leading to marine pollution. They are typically made of polyvinyl chloride, nylon, polyethylene terephthalate, polyethylene, polypropylene, polystyrene, polyvinyl alcohol, and polyamide (Napper *et al.*, 2015; Avio *et al.,* 2016; Carr *et al.,* 2016).

Microplastic pollution is a rising global concern due to its distribution, persistence, and adverse effects to the ecosystem. The concentration of microplastics reached up to 2500 particles km^{-2} in global marine regions, including the Arctic region, Northwest Atlantic, Northeast Atlantic Ocean, Southern North Sea, Mediterranean Sea, and the East China Sea (Auta *et al.,* 2017). Microplastics have not only polluted multiple geographical regions, they also persist as petrochemical-based microplastics are continuously used in various products for functionality. Microplastics also act as a carrier that adsorbs and bioaccumulates environmental pollutants within living organisms, thereby increasing the bioavailability of pollutants that are eventually biomagnified in the tissues of organisms at higher levels of the food chain,

such as humans. The global environmental concern regarding plastic waste has diverted attention towards the development of bio-based biodegradable plastics.

Previous reports have revealed microplastic ingestion by zooplankton (Md Amin *et al.*, 2020) whereby they are capable of varied microplastic uptake depending on the size of the microplastics, the life stage of the copepod and the species of the copepod. Microplastics are ingested by zooplankton through filter-feeding and egested in faecal pellets, generally within several hours (Barnes *et al.,* 2009; Cole *et al.,* 2013). It is imperative to study the ingestion and egestion of microplastics by planktonic organisms as they are at the base of the food web, hence they could bio-magnify chemical contaminants associated with microplastics to subsequent trophic levels (Turner, 2004). The types of zooplankton reported to ingest microplastics include holoplankton (Copepoda, Tunicata, and Euphausiacea), meroplankton (Mollusca and Decapoda), and microzooplankton (Dinoflagellata) (Cole *et al.*, 2013).

Copepods are among the most studied organisms related to the environmental impacts of microplastics (Cole *et al.,* 2013; Cole & Galloway, 2015). As the effects have been shown to be species-specific, there has not been any previous study regarding the ingestion of microplastics by the copepod *Nitokra lacustris pacifica*. However, *Nitocra spinipes*, a copepod from a closely related genus, was screened for acute toxicity of leachate from weathering plastics. Leachate from 8 out of 21 types of plastics induced acute toxicity and mortality in *N. spinipes* before and/or after irradiation (Bejgarn *et al.,* 2015). A proper study on the feeding preferences of *N. lacustris pacifica* has not been reported. Furthermore, the size of conventional microplastics typically ingested by copepods are between 10 µm and 20 µm for polyethylene (Coppock *et al.,* 2019). The common types of microplastics ingested by copepods or used in copepod ingestion studies are polyethylene terephthalate, nylon, and polyethylene (Coppock *et al.,* 2019).

Polyhydroxyalkanoate (PHA) is a microbially synthesised, biodegradable, and biocompatible polymer with thermoplastic or elastomeric properties (Amirul *et al.,* 2008). The PHA material is chiefly produced by certain genera of bacteria fermented in low-nutrient-high-carbon conditions with applications in packaging, medical or pharmaceutical industries (Verlinden *et al.,* 2007). PHA types that exhibit different thermal and mechanical properties can be altered for specific applications by manipulating the strain, carbon source, growth conditions and the PHA synthase gene (Ntaikou *et al.*, 2018). Among the types of PHA, poly(3-hydroxybutyrate) [P(3HB)] is the most common and naturally occurring PHA, which is more biocompatible and easily processable than other PHA types. P(3HB) also has mechanical properties desirable for medical and pharmaceutical applications, such as the pericardial patch, cardiovascular stent and drug delivery (Williams & Martin, 2002). The increasing applications and commercial use of PHA require extensive research concerning the environmental impact of PHA-based products. Discarded PHA products also degrade into microplastics eventually. There are very few reports on PHA microplastics in the marine environment or the feeding of PHA microplastics to copepods. In this study, the PHA microbeads developed using P(3HB) were characterised and the effects of ingestion and egestion of the PHA microbeads by the harpacticoid copepod *N. lacustris pacifica* were evaluated.

Materials and Methods

Biosynthesis of P(3HB)

P(3HB) was produced by the bacterial strain *Massilia haematophila* UMTKB-2 (Genbank accession number KT025845) isolated from brackish water in Mengabang Telipot, Kuala Nerus, Terengganu, Malaysia (Kiun *et al.*, 2016). The strain subculture, glycerol stock, seed culture, nutrient rich (NR) medium, mineral salt medium (MSM) and trace elements were prepared, and then P(3HB) was produced according to the method done by Kiun and co-

workers (Kiun *et al.*, 2019). The cell biomass was obtained by measuring the optical density of the culture broth at a wavelength of 600 nm. Subsequently, the cell culture was centrifuged twice at 4ºC and 9,000 rpm for 10 min. The cell pellet was stored at -80ºC overnight before being freeze-dried at -45ºC for 72 h using a freeze-dry machine (Labconco, USA). Gas chromatography was carried out to determine the composition of the PHA produced (Yatim *et al.*, 2017).

Extraction of P(3HB)

The polymer P(3HB) was extracted (Figure 1) as previously described by Kiun and co-workers (Kiun *et al.*, 2019). Freeze-dried cells were stirred with chloroform ($CHCl_3$) for 48 h to disrupt the cells and extract P(3HB) (Amirul *et al.*, 2008). The inactivation and removal of endotoxin were achieved by using hydrogen peroxide, after which the endotoxin level was tested using E-TOXATE™ Kits (Sigma Aldrich, USA) (Rao *et al.*, 2010; Govindasamy *et al.*, 2019).

The Development of PHA Microbeads

The development of PHA microparticles in the form of microbeads (Figure 1) was carried out using the double emulsion solvent evaporation technique as described by Govindasamy and co-workers (Govindasamy *et al.*, 2019). The microbeads were extracted using the suction filtration technique with 0.2-µm nylon-66 filter papers, and then washed three times with distilled water and dried overnight for scanning electron microscope analysis (Govindasamy *et al.*, 2019).

In-vivo Evaluation of PHA Microparticle on Copepod

The copepod *N. lacustris pacifica* was cultured and maintained in the laboratory under 12 h light and 12 h dark cycle feed with microalgae *Nannochloropsis* sp. The first generation of adult *N. lacustris pacifica* was sorted and left unfed in filtered seawater for 24 h. The copepods were then subjected to two different treatments; fluorescent PHA microbeads (dyed with Nile Red (1 mg/mL in acetone)) with a concentration of ~700 PHA microbead mL^{-1} prepared in i) filtered seawater and ii) unfiltered seawater. This selected concentration of microbeads was in the scope of a laboratory-based study without exceeding the extreme concentration used in ecotoxicological studies (Lee *et al.,* 2013; Cole *et al.,* 2015). Filtered seawater was screened with Whatman GF/F filter membranes (47 mm diameter; 0.7 µm pore size) to remove suspended particle while unfiltered seawater was produced by filtering through a 20-µm mesh net to remove any zooplankton. These were conducted to determine if the availability of suspended particle will significantly affect the PHA ingestion of the copepods. Both treatments were further added with 300 µg C L^{-1} of *Nannochloropsis* sp. Live copepods were individually sorted and placed in 5.5-cm petri dishes (10 individual per replicate; 9 replicates per treatment) filled with 10 mL of the above treatment.

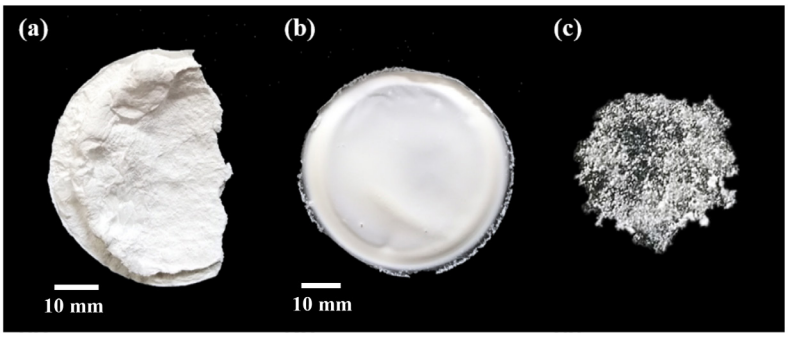

Figure 1: (a) Crude P(3HB) extracted from *M. haematophila* UMTKB-2, (b) PHA in the form of P(3HB) cast film and (c) PHA microbeads developed using the double emulsion solvent evaporation technique

To test the microbead ingestion, copepods were first exposed for 24 h with the respective treatment. To see the differences in egestion time after ingestion and the presence of microbeads in faecal pellets, the copepods were removed from the initial treatment and transferred to a petri dish filled with filtered seawater. About 5 drops of formalin were added into the petri dish to euthanise the copepods prior to the observation. The experiment was stopped when no PHA microbeads were detected in their bodies and faecal pellets. The egestion time and microplastic incorporation in the pellets were then calculated based on the percentage of the microbead presence over the total individual/ pellets tested by taking 3 replicates (n=30) per treatment every 24 h for further observation (Lee *et al.,* 2013; Cole *et al.,* 2015). The observations on the microbeads in both body and faecal pellets were done using the IX81 Inverted Fluorescence Microscope (Olympus, Japan). Statistical analysis was run using the software IBM SPSS Statistics 23.0. The Kruskal-Wallis test was used to compare the percentage of PHA microbeads ingested with the faecal pellets between filtered and unfiltered treatments.

Results and Discussion

The Characterisation of PHA and PHA Microbeads

M. haematophila UMTKB-2 produced 0.744 g/L of P(3HB) using a carbon:nitrogen (C/N) ratio of 50 and glucose as the sole carbon source. The endotoxin level of the PHA microbeads after the removal of the former was below the minimum safety threshold. Strain UMTKB-2 was observed to produce a higher P(3HB) yield than other species of the same genus. A glucose-utilising seaweed isolate, *Massilia* sp. UMI-21, utilised a C/N ratio of 60 to produce P(3HB) with a concentration of 0.02 ± 0.01 g/L, which was less than the P(3HB) produced by strain UMTKB-2 despite using a higher C/N ratio (Han *et al.*, 2014). Among other seven PHA-producing bacteria of the *Massilia* genus, which were *Massilia albidiflava*, *Massilia lutea*, *Massilia dura*, *Massilia plicata*, *Massilia brevitalea*, *Massilia aerilata* and *Massilia aurea*, the highest P(3HB) concentration obtained was 1.25 g/L from *M. lutea* using 4 g/L of soluble starch as the sole carbon source (Cerrone *et al.*, 2011).

Microbeads are particulates commonly suspended in surfactants to mechanically exfoliate and cleanse the face or body. Microbeads are manually massaged into the skin to dislodge shedding outer epidermis, improve skin tactile softness and visual smoothness, as well as promote the absorption and application of cosmeceutical or skincare products (Draelos, 2019). The PHA microbeads developed were spherical-shaped and possessed a heterogenous average size range of 10.1 µm to 140 µm with an average diameter of 38.44 µm (Figure 2). The size of commercial exfoliating microbeads made from petrochemical-based plastic ranges from 10 to 500 µm, while the size of human skin pores ranges from 250 to 500 µm (Flament *et al.*, 2015; Napper *et al.*, 2015). Furthermore, according to reports on Japanese coastal waters, the typical size of microbeads found in the environment was less than 2 mm, whereby microplastics between 300 µm and 400 µm in size were dominantly microbeads. Additionally, 10% of the microplastics were microbeads (smaller than 800 µm) (Isobe, 2016; Tanaka & Takada, 2016). The size of the PHA microbeads produced was more compatible with the size of human skin pores than the sizes of the PHA microbeads reported by Mohamed and co-workers that ranged from 0.3 µm to 0.6 µm (Mohamed *et al.*, 2017), and those of Murueva and co-workers that ranged from 0.7 µm to 2.6 µm (Murueva *et al.*, 2013). The heterogenous size of the PHA microbeads supplied to the microbead treatment setup is more akin to the natural seawater environment than the size of controlled microbeads.

The material P(3HB) was the chosen PHA type used to produce the microbeads in this study as P(3HB) is the most commonly found and studied polymer of the PHA family (Yatim *et al.,* 2017; Amelia *et al.,* 2019). Further study of the ingestion and egestion of other types of PHA by plankton in different environmental conditions is recommended.

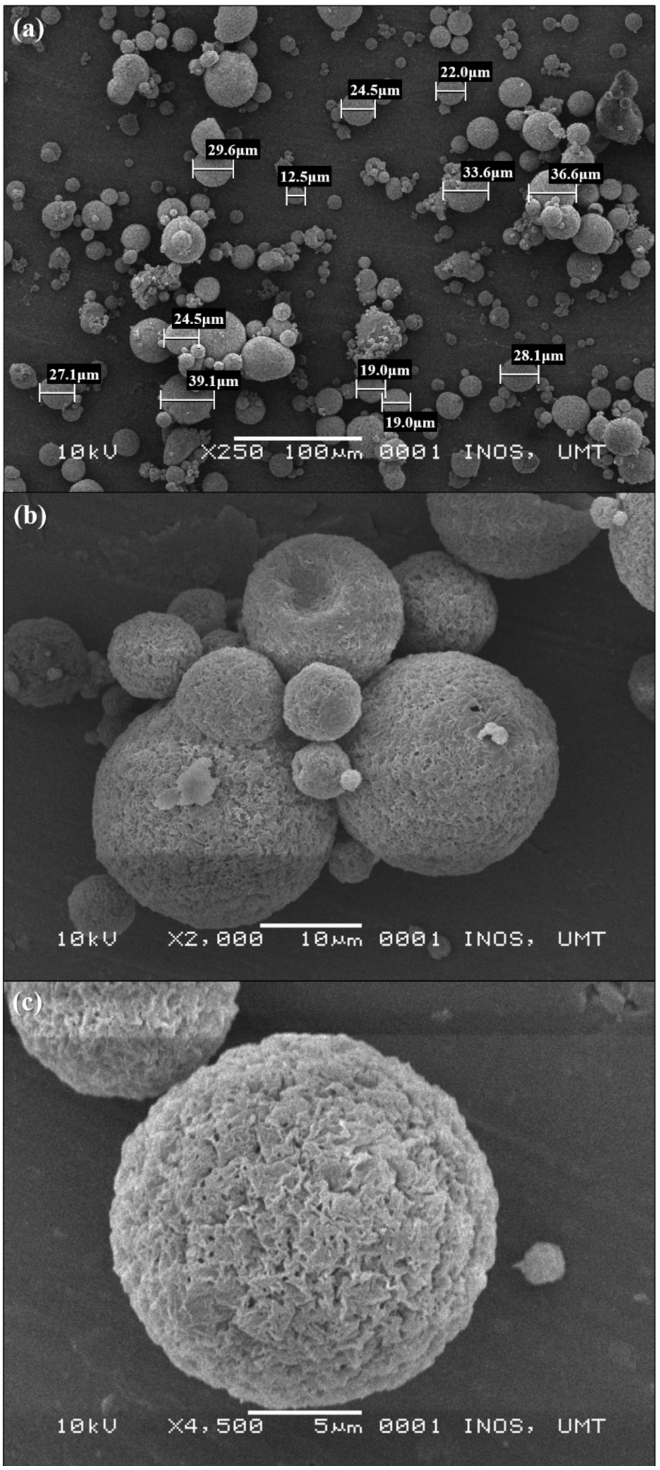

Figure 2: Scanning electron microscope (SEM) images of PHA microbeads with (a) 250x, (b) 2000x and (c) 4500x magnifications

The Presence, Ingestion and Egestion of PHA Microbeads by Copepod

The fluorescence of the Nile-red-stained PHA microbeads in *N. lacustris pacifica* confirmed the presence of soluble PHA in the tissue of the living organism. The PHA microbeads were observed in the digestive tracts and faecal pellets of *N. lacustris pacifica* (Figures 3), confirming the ingestion and egestion of the PHA microbeads by the copepods after being exposed for 24 h.

In both filtered and unfiltered seawater treatments, the PHA microbeads ingested by *N. lacustris pacifica* was > 70% and the egestion time to fully remove the microbeads from the body required only 3 days (Figure 4a). Similarly, the percentage of fluorescent PHA microbeads in faecal pellets followed the same trend of decline from the first day until the third day of exposure (Figure 4b). However, no significant difference on the percentage of PHA microbeads ingested and in faecal pellets were observed between filtered and unfiltered seawater treatment ($p > 0.05$).

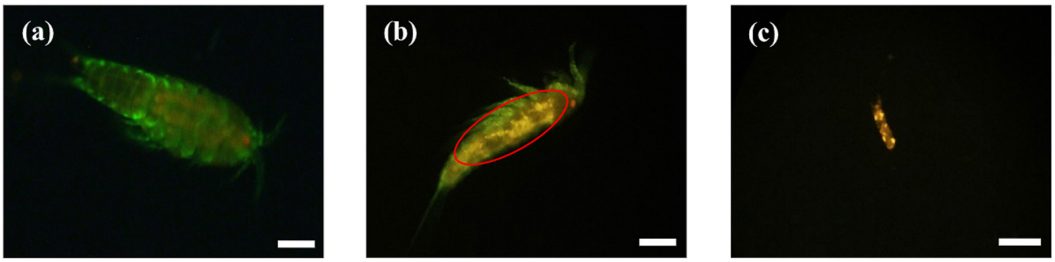

Figure 3: Microscopic images under an inverted fluorescence microscope of *N. lacustris pacifica*; (a) The absence of the PHA microbead in the digestive tract, (b) the presence of the PHA microbead indicated in the red circle, (c) the PHA microbeads in a faecal pellet. [Scale bar: Copepods = 200 µm; faecal pellet = 100 µm]

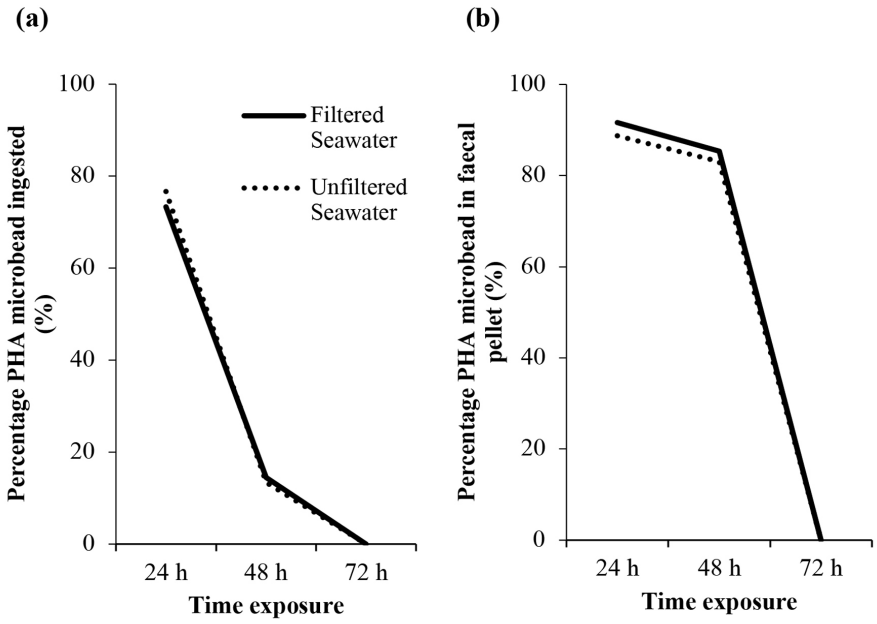

Figure 4: (a) The percentage of PHA microbeads ingested by *N. lacustris pacifica* (n=30). (b) The percentage of PHA microbeads in the faecal pellets of *N. lacustris pacifica* within three days of exposure. No mortality was observed in this short-term exposure

Cole *et al.* (2013) reported a high percentage (> 70%) of microbeads (7000 particle mL^{-1}; 7.3 µm polystyrene) ingested by copepods *Acartia clausi, Calanus helgolandicus, Centropages typicus,* and *Temora longicornis* after an exposure of 24 h. They also demonstrated that dose-dependent microplastic exposure could significantly reduce the algal ingestion rate of the copepod *Centropages typicus*. Similar results were also observed in copepod *Tigriopus japonicus* tested using polystyrene beads at a higher concentration (5.25×10^5 (6 µm) – 9.1×10^{11} particle mL^{-1} (0.05 µm)) and the presence of beads in faecal pellets was observed after an exposure of 24 h (Lee *et al*., 2013). Using a lower particle concentration, the ingestion of polystyrene microbeads (50 – 200 particle mL^{-1}, 15 µm) by copepod *Calanus finmarchicus* was reported to be higher among more than 80% of the overall individuals after 24 h, while egestion was recorded to be faster than the current study of among 94% of individuals within 4 h, while the other 6% of individuals egested faecal pellets within 18 h (Vroom *et al.*, 2017). Likewise, Dedman (2014) showed that copepod *Calanus typicus* ingested 100% of microbeads at a concentration of 100 particles mL^{-1}, whereas 100% of individuals showed an adherence of microbead to the outer part of the body after an exposure of 24 h. The difference in copepod response after ingestion could be due to species-specific effects, the variations of material and the concentration of microbeads used in the experiment. According to Straub and co-workers (Straub *et al*., 2017), petroleum-based microplastics have relatively lower ingestion rates, hence a lower assimilation efficiency compared with PHA microplastics, but both microplastic types led to a lower wet weight gain among amphipods. Both microplastic types caused digestive constraints in the amphipods (Straub *et al*., 2017). Although higher ingestion rates may indicate a better assimilation efficiency, they also lead to a more rapid consumption of microplastic-contaminant complexes in the environment over time.

Conclusion

Zooplankton *N. lacustris pacifica* was confirmed to consume PHA microbeads as food, as well as proven to uptake and egest these microbeads with possible assimilation. Data revealed that different seawater treatments did not affect the ingestion and egestion by *N. lacustris pacifica* throughout the analysis. The proven uptake of micro-sized biodegradable plastics by *N. lacustris pacifica* poses new unanswered research questions, such as the potential harm or lack of it when micro-sized biodegradable plastics, such as PHA, polylactic acid (PLA), starch blends and cellulose-based plastics, are consumed by zooplankton. The chronic exposure of microplastics to several copepod generations, with selected range of microbead size and concentration, are needed to study the effect of biodegradable microplastics on copepod mortality.

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