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Original Article

Evaluation of polyethylene microplastic bio-accumulation in hepatopancreas, intestine and hemolymph of freshwater crayfish, *Astacus leptodactylus*

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Abstract: Microplastics (MPs) are one of the biggest environmental problems threatening aquatic life. The accumulation of MPs in the body of aquatic animals can play a role in transferring these pollutants into the food chain. These pollutants can significantly affect the physiology of aquatic animals. In this study, the bioaccumulation capability of MPs in the body of freshwater crayfish, *Astacus leptodactylus* has been evaluated. For this purpose, crayfish were exposed to 0, 500, and 1000 µg L^{-1} of polyethylene MPs (PE-MPs) for 28 days. Then, the accumulation of MPs in hemolymph, hepatopancreas, and intestine of crabs was investigated by Fourier transform infrared spectroscopy (FTIR). Bioaccumulation of PE-MPs in the hemolymph, hepatopancreas, and intestines was observed in the crayfish exposed to PE-MPs. This study showed that FTIR is a suitable method for identifying and measuring MPs in aquatic organisms.

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Introduction

The increase in the global production of plastics has caused a large amount of plastic waste to be released into the environment every day. Wastes and plastic wastes eventually enter aquatic ecosystems through washing or sewage. Therefore, aquatic ecosystems, especially seas and oceans, are the centers of the accumulation of plastic waste in the world. Plastic waste's physical and chemical decomposition causes the larger pieces to be broken into smaller pieces (Chamas et al., 2020). From this point of view, microplastics (MPs) of different sizes are one of the most obvious plastic pollutants in aquatic ecosystems (Guzzetti et al., 2018; Alimba and Faggio, 2019; Strungaru et al., 2019; Prokić et al., 2021). MPs can affect biological dynamics and biodiversity in aquatic ecosystems. The change in physical and chemical characteristics and the release of plasticizers from MPs have caused an increase in their risk compared to virgin plastic polymers (Karami et al., 2016; Liu et al., 2020). In recent years, the various types of MPs, including polystyrene (PS), polypropylene (PP), polyethylene (PE), polyethylene terephthalate (PET), polyvinyl chloride (PVC), and polyamide (PA) in different forms, have been reported in aquatic ecosystems (Erni-Cassola et al., 2019; Schwarz et al., 2019). Polyethylene (PE) has been classified as a major source of MPs in aquatic and terrestrial environments (Wang et al., 2018; Sun et al., 2021). Despite the variety of available polymers, about 45% of global production is made with polyethylene.

MPs can enter the body of aquatic animals through the digestive or respiratory system and are distributed in the body through the blood (Banaee et al., 2021; Dey et al., 2021). The exposure of aquatic organisms to plastic waste can have various consequences (Paul-Pont et al., 2016; Chen et al., 2020; Banihashemi et al., 2022). Metabolic damage, oxidative stress, dysfunction of the immune system (Espinosa et al., 2017, 2019; Tang et al., 2018; Banaee et al., 2019b;) and change in intestinal microbial diversity (Lu et al., 2018), growth inhibition (Besseling et al., 2014; Au et al., 2015), reduced growth (Della Torre et al., 2014), reduced feeding activity (De Sá et al., 2015) and

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abnormal behavior (Rist et al., 2016), physical damage in the digestive system (scratching, perforation, and obstruction), hepatotoxicity (Nematdoost Haghi and Banaee, 2017; Huang et al., 2021) and bioaccumulation of MPs in various tissues (Dey et al., 2021) have been reported in aquatic animals exposed to MPs.

Crustaceans, as aquatic scavenger organisms, may expose to MPs. Thus, these animals are an excellent indicator to assay MPs effects. Due to its feeding habits, the freshwater crayfish is one of the best biological indicators for monitoring the pollution of aquatic ecosystems (Hong et al., 2018; Banaee et al., 2019a, 2020). Therefore, the biological response of these crustaceans to environmental pollution can reflect the health status of aquatic ecosystems. Narrow-clawed crayfish, *Astacus leptodactylus* (Astacidae: Malacostraca), is a freshwater crustacean found naturally and widely in some lakes, pools, and rivers in northwestern Iran. In recent decades, this species has been introduced to many dams and internal lakes of Iran. Therefore, *A. leptodactylus* was selected as a model organism in this study and exposed to different concentrations of MPs to understand the effect of MPs on it.

Materials and Methods

This study was conducted from September to December 2021 at the Department of Aquatic Health and Diseases, Faculty of Veterinary Medicine, Shiraz University, Iran. The Birjand and Shiraz Universities' Animal Ethics Committee approved all experimental procedures.

Freshwater crayfish, *A. leptodactylus* of both sexes weighing 42.11±0.31 cm and length 10±6.23 g, were caught from local waters (Heft Brom, Shiraz) and transported to the laboratory. Before starting the experiment, the crabs were adapted to the laboratory conditions (15±2°C, pH 7.2±0.3, with dissolved oxygen 8.2±0.6 mg/L, and under a photoperiod (14 light: 10 dark) cycle, electrical conductivity 693.85±174 µS cm and salinity 0.3±0.102 g/L) for two weeks. During the acclimatization period, the crayfish were fed formulated shrimp feed (Beyza Feed Company, Shiraz, Iran: 45-55% protein, 10-11% lipid, 20-30% carbohydrate, 1.5-2% fiber), twice per day. The surplus food and fecal matter were removed from each aquarium, and water was renewed daily by adding fresh water.

Ninety adult crayfish were randomly introduced to nine aquariums (10 crayfish per aquarium) to carry out three experimental treatments (with three independent replicas). The crayfish were divided into three experimental groups and exposed to 0.0, 500, and 1000 $\mu g.L^{-1}$ PE-MPs for 28 days. During the experimental periods, crayfish were fed two times daily, while crayfish were starved for one day before taking the sample.

After the experimental procedure, 12 crayfish from each treatment were sampled and anesthetized on ice. Hemolymph was acquired from the sternal artery by a sterile syringe containing Alsever's solution adjusted for *A. leptodactylus* as the anticoagulant (Banaee et al., 2019). After the autopsy, the hepatopancreas of the crayfish was separated and placed in liquid nitrogen. Next, the hemolymph and hepatopancreas samples were then dried in a freezer dryer. Subsequently, polyethylene (MPs) was detected in the tissues by FTIR Spectrometer (Bruker). FTIR could detect the high density of polyethylene in the 1445-1650 wavenumber (cm^{-1}) (Li et al., 2022).

Results

FTIR results are presented in Figures 1-3. The FTIR spectrum showed the presence of polyethylene MPs in the hemolymph and hepatopancreas of crayfish. The change in FTIR peaks in the wavenumber range of 717, 1600, 1680, and 2928 (cm^{-1}) indicated the presence of polyethylene MPs. Furthermore, these results prove that MPs could penetrate other vital organs such as the hepatopancreas after entering the hemolymph. The difference between the FTIR peaks in the experimental groups treated with different amounts of MPs and the standard may indicate the existence of differences in the concentration of MPs in hemolymph and hepatopancreas.

FTIR results are presented in Figures 1-3. The FTIR spectrum showed the presence of polyethylene

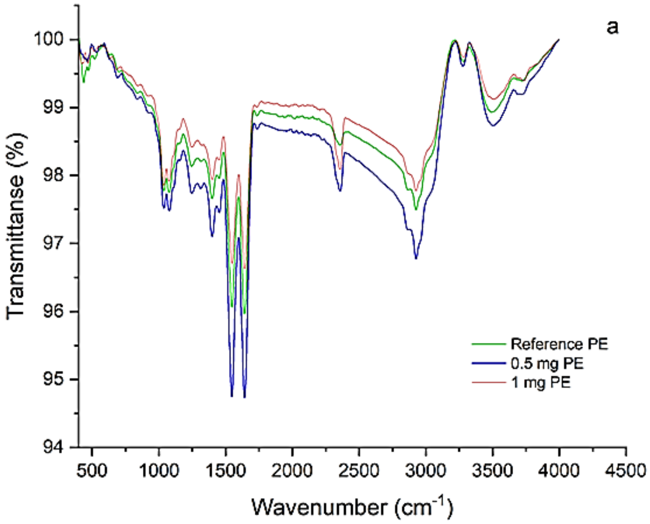

Figure 1. Fourier infrared spectroscopic spectrum related to hemolymph (a) samples to detect PE-MPs.

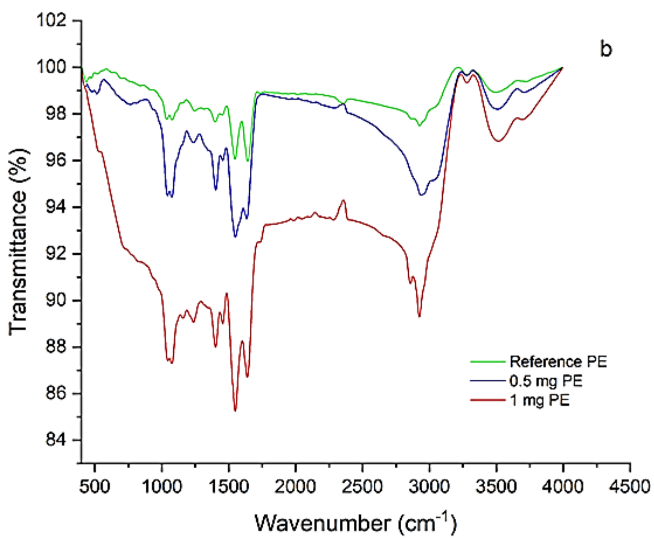

Figure 2. Fourier infrared spectroscopic spectrum related to hepatopancreas (b) samples to detect PE-MPs.

MPs in the hemolymph and hepatopancreas of crayfish. The change in FTIR peaks in the wavenumber range of 717, 1600, 1680, and 2928 (cm^{-1}) indicated the presence of polyethylene MPs. Furthermore, these results prove that MPs could penetrate other vital organs such as the hepatopancreas after entering the hemolymph. The difference between the FTIR peaks in the experimental groups treated with different amounts of MPs and the standard may indicate the existence of differences in the concentration of MPs in hemolymph and hepatopancreas.

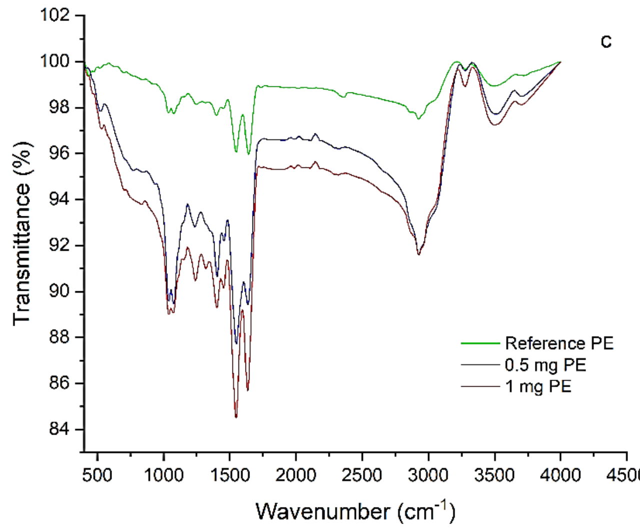

Figure 3. Fourier infrared spectroscopic spectrum related to intestine (c) samples to detect PE-MPs.

Discussions

The spectra of the polyethylene obtained in disk form are shown in Figures 1-3. The results of this study showed that exposure of crayfish to MPs for 28 days led to the accumulation of MPs in the hemolymph, intestine and hepatopancreas (Wang et al., 2020; Kim et al., 2021) Fourier-transform infrared spectroscopy (FTIR) is a well-known analytical tool that can detect organic substances' functional groups and molecular structure (Rytwo et al., 2015; Lohumi et al., 2017; Litvak et al., 2018). Li et al. (2022) characterized the FTIR bands as the functional groups related to polyethylene (Table 1). The band I and II at 721-993 cm^{-1}, indicated the existence of aromatics derivatives. The band III and IV at 950-1300 cm^{-1} demonstrated phenols, alcohols, and ethers groups. The results showed a significant transmittance pattern in the region of 1455-1646 cm^{-1} (band V and VI) (Gulmine et al., 2002; Xu et al., 2019; Daniel et al., 2020; Daniel et al., 2021). These peaks belong to the Light aromatics and aromatic ring (aryl). The band VII at 2919 cm^{-1} showed methylene C-H asymmetric stretch groups. Different chemical bonds and structures of various plastics show unique spectra through FTIR spectroscopy. Since MPs are composed of carbon-based functional groups linked by covalent bonds, these analytical tools can identify all types of plastics (Mecozzi et al., 2016).

Table 1. Studies of the FTIR bands to the functional groups and possible derivatives (Li et al., 2022).

Wavenumber (cm^{-1})	**Possible derivatives**
4000–3500	Water, Phenols, Alcohols
3620–3540	Tertiary alcohol, OH stretch
3095–3075	Terminal (vinyl) C-H stretch; Hydrocarbons
2935–2915	Methylene C-H asymmetric stretch
3100–2600	Hydrocarbon
2400–2260	CO_2 (carboxylic stretch)
2240–2060	CO
2250–2000	Oxygen-contenting compounds
1840–1590	Ketones, Aldehydes, Esters, Carboxylic acids, Primary amides
1680–1620	Aromatic ring stretch
1650–1450	Light aromatics
1485–1445	Aromatic ring (aryl)
1300–950	Phenols, alcohols, ethers
1225–950	Aromatics
920–900	Mono-substituted alkenes
750–710	Methylene $-(CH_2)_n-$ rocking ($n > 3$) or meta di-substituted aromatics
900–670	Aromatics
730–630	CO_2

The bio-accumulation of MPs was reported in the visceral mass and haemolymph of the bivalve (*Amarilladesma mactroides*) and mussels (*Brachidontes*. *Rodriguezii*) (Truchet et al., 2021). Also, Fibre of MPs was detected in the whole body of *Crangon crangon* (Devriese et al., 2015), *Pandalus borealis* (Fang et al., 2018), *Paratya australiensis* (Nan et al., 2020), *Fenneropenaeus indicus* (Daniel et al., 2020), and in the stomach and foregut of *Plesionika narval* (Bordbar et al., 2018), *C. crangon* (McGoran et al., 2018), and *Macrobrachium rosenbergii* (Li et al., 2021). Bio-accumulation of MPs was reported in the digestive system of crustaceans (McGoran et al., 2018; Bordbar et al., 2018; Carreras-Colom et al., 2018), the whole body (Daniel et al., 2020), visceral organs (Carreras-Colom et al., 2020), soft tissue (Nakao et al., 2020; Daniel et al., 2021), gills (Zhang et al., 2019; Not et al., 2020), hepatopancreas (Martinelli et al., 2021; Zhang et al., 2022), and hemolymph (Zhang et al, 2022).

As conclusion, polyethylene MPs can be absorbed in the gills and intestines and enter the hemolymph. Then MPs are distributed through hemolymph in different body tissues and may accumulate. Therefore, the measurement of PE-MPs by the Fourier transform infrared spectroscopy method of chemical imaging, i.e., FTIR, is useful for MPs to be automatically analyzed.

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