CHAPTER 3

MATERIALS AND METHOD

3.1 Sample collection

Samples of cockles (*Anadara granosa),* mussels *(Corbicula leana)* and coastal water were collected at the sea located at Tanjung Karang, Selangor, and Sebatu, Melaka. Coastal water consists of seawater where the samples of cockles and mussels were cultivated. A total of two kg each and three litres of sea were collected from the sea to screen and identify the microplastics. The cockles and mussels from Tanjong Karang, Kuala Selangor were harvested at area around 5 km radius from the seashore on March, July and October 2021 as peak season for the growth of cockles and mussels. The coordinate of the sampling sites for each of the samples were recorded. Table 3.1 and Figure 3.1 below shows the coordinate and the location of the cockles and mussels collected at the cultivation site.

Table 3.1: Coordinate of the cockles and mussels sampling site

Samples	**Samping site**			
	Tanjong Karang, Selangor		**Sebatu, Melaka**	
	Latitude	**Longitude**	**Latitude**	**Longitude**
Cockles	3°13’29.8’’N	101°17’19.4’’E	2°05’59.7’’N	102°27’16.4’’E
Mussels	3°14’41.3’’N	101°17’34.3’’E	2°06’43.4’’N	102°27’16.4’’E

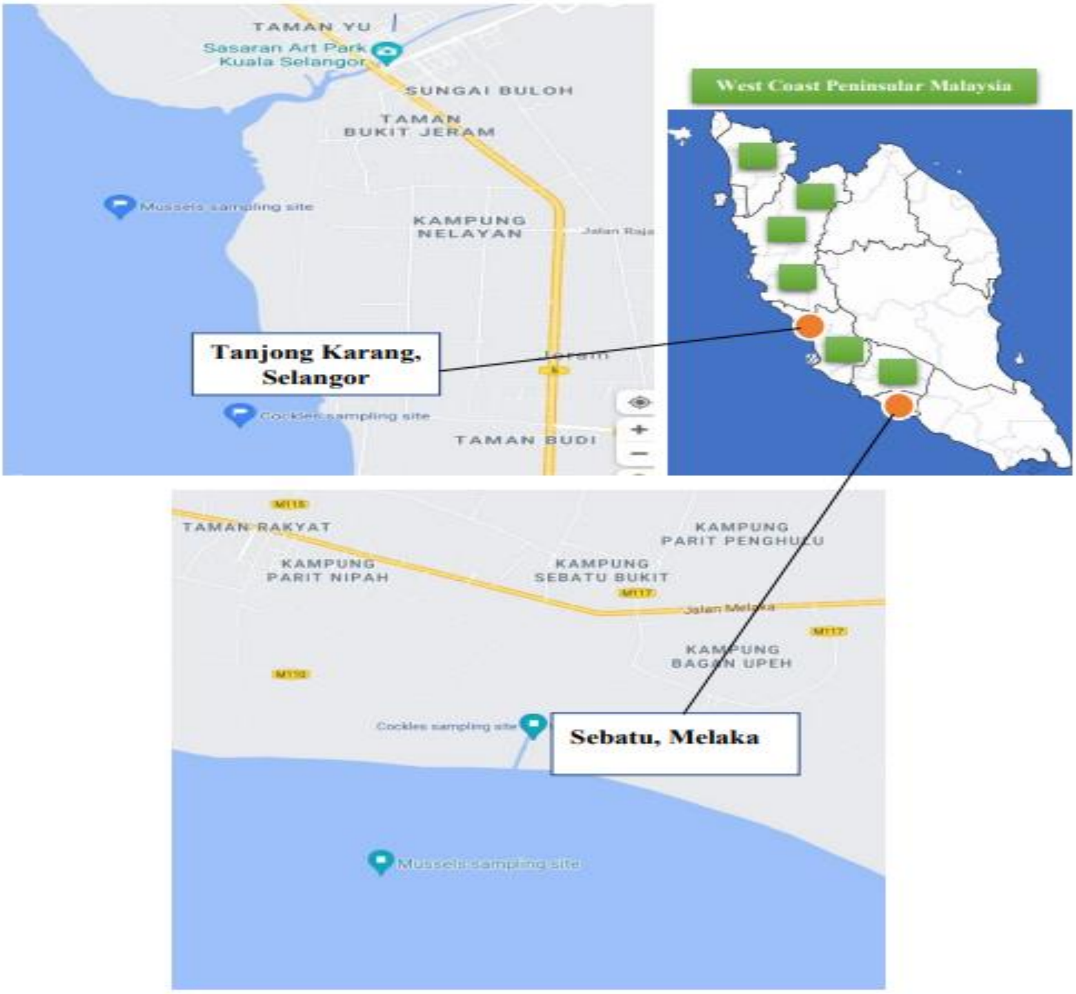

Figure 3.1: Sampling sites of cockles and mussels from two strategic sites at West Coast Peninsular Malaysia

All samples were stored in a polystyrene box with dry ice pack and the temperature was maintained below 0 ^{O}C during transportation. Following collection, after arrival at the laboratory of the Faculty of Science and Technology, USIM, Nilai, about half of the samples of cockles and mussels were shucked and immediately processed prior to physical analysis while the remaining samples were packed in the aluminium pouch and stored at the -20 ^{O}C for further analysis. Meanwhile, coastal water was stored in the glass container at -20 ^{O}C. All analysis were done in triplicates.

3.2 Microplastic screening on cockles, mussels and seawater samples

3.2.1 Assessment of microplastics in mussels and cockles

The assessment of microplastics in mussels and cockles were according to Espiritu et al. (2019) with slight modification. The weight and length of the remaining mussels and cockles were measured before shucking in triplicates, each consisting of 30 g of shucked cockles and mussels. The cockles and mussels were placed in separate conical flasks followed by oven-drying at 60 °C for at least 2 hours until all the water dried out. After oven-drying, 20 mL of concentrated nitric acid (HNO_3) (Merck, Germany) was added to each sample, which then were left to dry for at least two days until no more organic materials left out and have reached its optimum digestion. Subsequently, each sample was heated over a hot plate at temperature ranging between 80–90 °C for 20 minutes. Then, 20 mL of warm distilled water (80 °C) was added to each conical flask prior to dilution. The samples were then subjected to vacuum filtration (Whatman Grade GF/C). The filters obtained were eventually placed on glass Petri dishes and oven dried at 50 °C within one to two hours until the samples dried. The dried samples on the filter papers were then observed under a light and fluorescence microscope (magnification: 40x) for the detection of potential microplastics' particles. Identified microplastics were documented and characterized using the description based on location and sample types as portrayed in Table 3.2.

3.2.1 Assessment of microplastics in seawater samples

The assessment of microplastics presence in coastal water sample were conducted according to Espiritu et al. (2019) with slight modification. The samples were vacuum filtered with distilled water using filter paper. The collected solids were placed in pre-weighed beakers prior to oven-drying at 90 °C until they achieve a constant weight (+ 0.0001 g).

The potential microplastic particles obtained from the oven-dried method were undergo wet peroxide oxidation (WPO) to digest the remaining organic materials and to extend the microplastics isolation. This was conducted by adding 20 mL of 0.05 M aqueous ferrous sulphate ($FeSO_4$) (R&M, Malaysia), followed by 20 mL of 30% hydrogen peroxide (H_2O_2). Following that, the mixture was left at room temperature for 5 minutes to allow the reaction to complete and the mixture to homogenize. Subsequently, the mixture was stirred and heated over a hot plate at 75 °C until the bubbles formed. After the formation of bubbles, the sample was put aside from the hot plate to allow cooling down, letting the bubbling to subside. The heating and cooling cycle were repeated for 30 minutes additionally. Supplemental 5 mL of 30% H_2O_2 was be added to the solution if needed until there was no organic material left.

Sodium chloride (NaCl) (Merck, Germany) was added into the mixture to incline the density separation of the aqueous solution with a ratio of 6:20 (6 g per 20 mL). Then, the resulting mixture was incubated in a shaking incubator for 15 min (50 °C at 100 rpm) or until the salt is homogenously dissolved in the solution. The mixture was placed into a beaker and left for the reaction to complete overnight and cause density separation (Masura et al. 2015). The floated particles were filtered and collected followed by examining the potential microplastics under a light microscope (magnification: 40x). The observations on the microplastics were characterized according to Table 3.2 by Hidalgo-Ruz et al. (2012). The particles were then placed in a glass vial and further oven-dried at 40 °C to reduce sample noise prior to FTIR analysis. Table 3.2 below shows the characterization of microplastics by mean of physical analysis according to Hidalgo-Ruz and co-authors (2012).

Table 3.2: Characterization of microplastics according to Hidalgo-Ruz et al. (2012).

Characteristics	**Description**
Source	Consumer product degradation and raw industrial fragment
Type	Plastic pellet, fragment, plastic film, filament, granule, foamed plastic and Styrofoam
Shape	*For fragment:* Rounded, subrounded, subangular, angular *For pellet:* cylindrical, flat, disk, ovoid, spheres *General:* irregular, degraded, elongated, rough and broken edges
Erosion	Fresh, linear fractures, irregular surface, un-weathered, and level of crazing (conchoidal fractures) weathered, incipient alteration, grooves, jagged fragments, subparallel ridges and degraded
Colour	White, crystalline, clear-white, orange, cream, blue, red, black, opaque, brown, pink, green, grey, yellow, tan and pigmentation

Source: (Espiritu et al., 2019)

3.2.3 Characterization of the microplastics isolated from the samples

The potential microplastics observed under light microscope were identified and characterized by size (relative to a 5 mm scale bar), composition (based on the results of the FTIR spectroscopy), type of particle, shape, erosion history, and colour using the characteristics shown in Table 3.2. The identification of the possible sources, shape, and erosion history of microplastics were also recorded.

3.2.4 Identification of microplastic by FTIR Analysis

Individual microplastics particles obtained from the physical analysis were isolated and stored in small glass vial prior to chemical analysis. The transmission spectrums were generated using a IRTracer-100 Fourier Transform Infrared spectroscopy (PerkinElmer, USA) using averaging 8 scans at 4 cm^{-1} resolution. The spectral range was set to 4000 to 600 cm^{-1}. The spectra obtained were compared with those microplastics standards of polyethylene, polypropylene and polystyrene spectrum obtained by FTIR spectroscopy.

3.5 Statistical analysis

Statistical data analysis was performed using Minitab Version 17 (Stat Inc, USA). Two-way ANOVA was conducted to analyse the significant level of microplastics distribution among locations and sample types. Differences of p-value ($p < 0.05$) were considered statistically significant. Principal component analysis (PCA) was performed using a data matrix of FTIR spectrum of functional groups presence on each sample from two strategic sampling sites being compared to polymer types of microplastics standards to evaluate the relationship of microplastics presence between types of samples and sampling sites. PCA analysis was performed using Unscrambler X Version 10.4 (CAMO, USA).

3.6 Quality control measures

To minimize error or airborne contamination throughout all steps of the experiment including sampling, handling, processing and analysis, safety measure needs to be focused (Murphy, 2018). Prior to prevent contamination, all liquids, such as distilled water, ethanol and nitric acid were filtered through cellulose nitrate membrane filters with mesh size of 0.45 µm (Zaki et al., 2021). All glassware needs to be rinsed with filtered distilled water and 70 % ethanol prior to use. All apparatus including petri dish must be made from non-plastic polymer to avoid contamination of plastic debris come in contact with the samples. The entire preparation and extraction procedure were performed under a fume hood except for microscope analysis and minimize the foot traffic to reduce the possibility of airborne microplastics. All the glassware from the preparation and extraction protocol were covered with aluminium foil to avoid airborne contamination.