



**UNIVERSITI PUTRA MALAYSIA**

***ISOLATION AND CHARACTERISATION OF MICROPLASTICS IN  
FISHMEALS***

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FISHMEALS**



**BY,**

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**This thesis submitted in fulfilment of the requirement for the degree of Bachelor  
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and Health Sciences, Universiti Putra Malaysia**

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## ABSTRACT

### ISOLATION AND CHARACTERISATION OF MICROPLASTICS IN FISHMEALS

DOROTHY UNING ANAK WATT @ GUNDIE

**Introduction:** Microplastics (MPs) are defined as plastics between 1 and 1000  $\mu\text{m}$  in size. Primary MPs are plastics that are manufactured to be a size of microscopic size such as skincare products and air blasting technology. Microplastics are abundant in the marine environment as a result of fragmentation of the plastics disposed into the sea. Microplastics had been mistaken as the food for the marine organisms as MPs may appear to look like preys. **Objective:** This study was conducted to investigate the presence of MPs in fishmeals manufactured in Malaysia. **Methodology:** Two different brands of fishmeals were purchased from manufacturers, digested and filtered. Isolation and characterisation of isolated particles were made according to the morphology and chemical composition. Morphology of isolated particles were investigated using visual characterisation whilst chemical composition (type of plastic polymer and additives) were conducted using micro-Raman spectroscopy. **Result and discussion:** The finding showed that there was significant difference of morphology, plastic polymers and additives between the brands. **Conclusion:** There was presence of MPs in the fishmeals and there are chances that these MPs can be transferred to the higher trophic level through ingestion of animals fed with MPs contaminated fishmeals, thus call for further study to improve understanding on the effect of MPs to human health in term of the physical and chemical impacts.

**Keywords:** Microplastics, fishmeals, additives, plastic polymers

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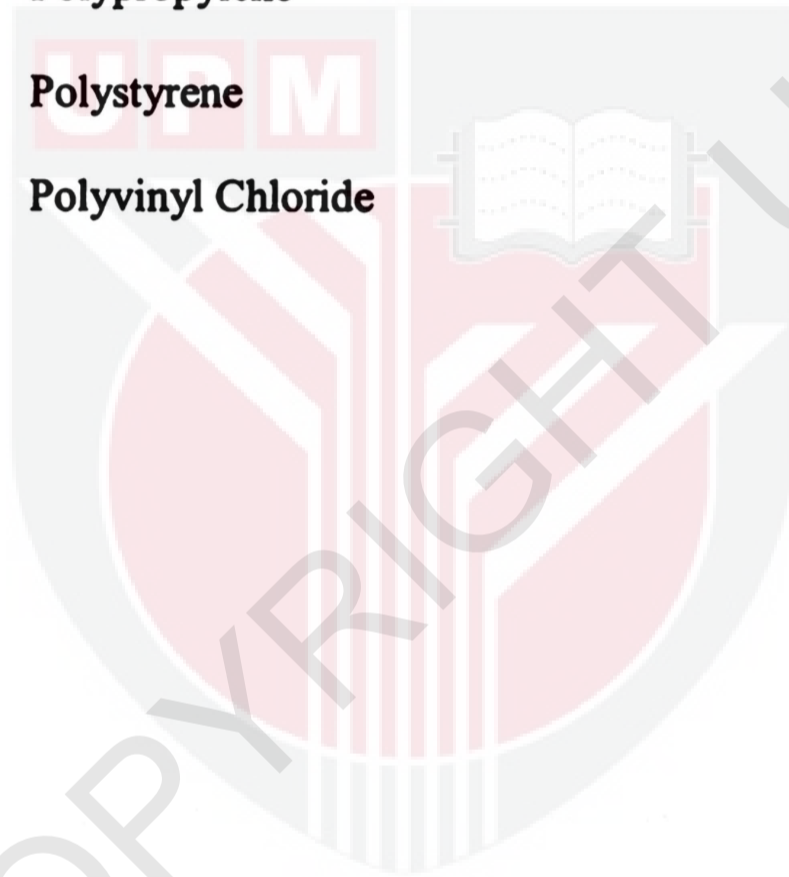
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## LIST OF ABBREVIATIONS

<b>FAO</b>	<b>Food and Agriculture Organisation</b>
<b>HDPE</b>	<b>High-Density Polyethylene</b>
<b>LDPE</b>	<b>Low-Density Polyethylene</b>
<b>PA</b>	<b>Polyamide</b>
<b>PC</b>	<b>Polycarbonate</b>
<b>PET</b>	<b>Polyethylene Terephthalate</b>
<b>PP</b>	<b>Polypropylene</b>
<b>PS</b>	<b>Polystyrene</b>
<b>PVC</b>	<b>Polyvinyl Chloride</b>



# CHAPTER 1

## INTRODUCTION

### 1.1 Background

Plastics are one of the great uses in the industry due to their beneficial characteristics such as light weight, great electrical insulator and efficient thermal. Thus, world plastics production has advanced numerously since the past decades from 1.7 million tonnes in 1950 and recorded up to 322 million tonnes in 2015 (PlasticsEurope, 2016). Despite these, plastics are non-degradable. Plastics end up become microplastics (MPs) as a result of natural degradation. Microplastics are defined as particles with a size 1 to 1000  $\mu\text{m}$  (Karami et al., 2016). It requires continuous mechanical action for plastics to fragment into MPs because of little UV exposure and cold temperature in the water unlike land (Barnes et al., 2009; Ryan et al., 2009).

Plastics are a significant concern to the environment and call forth the issue of MPs as surprisingly, 60 to 80% of marine debris comes from plastics (Moore, 2008). Whilst these figures are alarming, MPs was reported to be distributed along shorelines around the world as a result of ocean current and tides (Barnes et al., 2009). Assuming the current condition at its worst, there are considerable chances of debris from neighbouring countries surrounding Malaysia shorelines.

Microplastics caused disturbance in the food chain and bring upon threat to the marine organisms. Microplastics with the similar resemblance to food made the marine organisms to mistaken the MPs as food. In China, quantifiable MPs are found in the commercial bivalves (Li et al., 2015). The same finding of plastics ingestion also been seen in Columbia as they found MPs in the manila clams (Davidson & Dudas, 2016).

Besides, this has triggered a question on the existence of MPs in the fish products, especially those were made for either human or animals' consumption, such as fish meals, canned foods and salted fish. These worries started to garner rave attention from the researchers as previous studies found MPs inside the marine organisms. As an example of trophic transfer for MPs, Farrell & Nelson (2013) and Watts et al. (2014) showed their translocation from mussels to crabs.

Ingestion of MPs may induce both physical and chemical harm to human as MPs did to marine organisms (Rochman et al., 2015). However the effect is left unanswered as there is no scientific data and study is done to provide the evidences. In previous studies on marine organisms, the researchers had reported that ingestion of MPs caused limited food uptake as a result of blockage of feeding appendages and alimentary canal (Cole et al., 2013) and includes potential to be translocated into the intestine wall through the circulatory system (Browne et al., 2008). Microplastics also act as additive vector and may release these chemicals to the organisms (Rocha-Santos & Duarte, 2015; Rochman et al., 2015)

## **1.2 Problem statement**

Since the very first disclosure of MPs made by Thompson et al. (2004), there had been some reports on the abundance of MPs found in aquatic environments. Microplastics pose threat to the food web as fish, bivalves and other invertebrates mistaken the MPs as food. The findings have sparked the issue of the danger of MPs to its consumers as MPs capable to cause damage to the digestive tract of marine organisms (von Moos et al., 2012). Microplastics are capable to absorb and release chemical to the organisms (Barnes et. al., 2009; Kershaw et al., 2011; Thompson et al., 2004). The presence of MPs had been reported in gastrointestinal (GI) tract of marine organisms (Boerger et al., 2010; Lu et al., 2016; Rochman et al., 2015). In addition, MPs are most quantified in the gut and gills of fishes and had translocated to circulatory system and liver (Browne et al., 2008; Li et al., 2015; Lu et al., 2016; Setälä, Fleming-Lehtinen, & Lehtiniemi, 2014).

As fishing industries increases from year to year, the by-products of the fish such as fishmeals also grow massive due to the agriculture demand of feedstock. Some production of fishmeals is 'whole', that is, only water and some oil are extracted from the fish whilst some are made from fish leftover (FAO, 1986). Hence, there are chances of MPs to present in fishmeals. These fishmeals are used in the diets of poultry, pigs and fish which need prominent quality protein (FAO, 1986); in which these farmed animals end up on human plate.

Although there is limited information on the effect of the MPs on human, it can be assumed the similar incident may occur in human and affect their health either

through the direct ingestion of marine organism or indirect ingestion of poultry or pork consumed fishmeals contain MPs. As there is no available study on MPs abundance in fishmeals in Malaysia or other countries, the study is to justify the presence of MPs in fishmeals and with the following confirmation that if the fragmented forms also persist to exist in any environment; it deems a signal for further research in term of the effect of MPs toward human.



### 1.3 Study Justification

Several studies reported the occurrence of MPs in the gastrointestinal (GI) tract of fish. For example, Avio, Gorbi & Regoli (2015), found MPs in the stomach and liver of the fish mullet whilst in Lu et al. (2016), MPs are found in the gut and gills of zebrafish. In addition, MPs are also reported in the stomach of large pelagic fish (Romeo et al., 2015). These MPs accumulated and quantified in mainly gut and gills and the smaller MPs entered hepatic tissue via circulatory system (Lu et al., 2016). Such translocation of MPs may transfer MPs into edible tissue of fish. In Malaysia, fishmeals are extensively used as protein sources or feedstock for non-ruminants and aquaculture feeding (Wan Zahari & Wong, 2009) and theoretically, feeding poultry with contaminated fishmeals could translocate MPs into their flesh. Hence, the current study enables investigation to understand the MPs contamination in Malaysian marine ecosystems and marine organisms. Therefore, the current study enables investigation of the possible indirect transfer of MPs into the human body through ingestion of the MPs-contaminated farmed animals. Last but not least, the finding may be useful to generate baseline data for future research.

## 1.4 Conceptual Framework

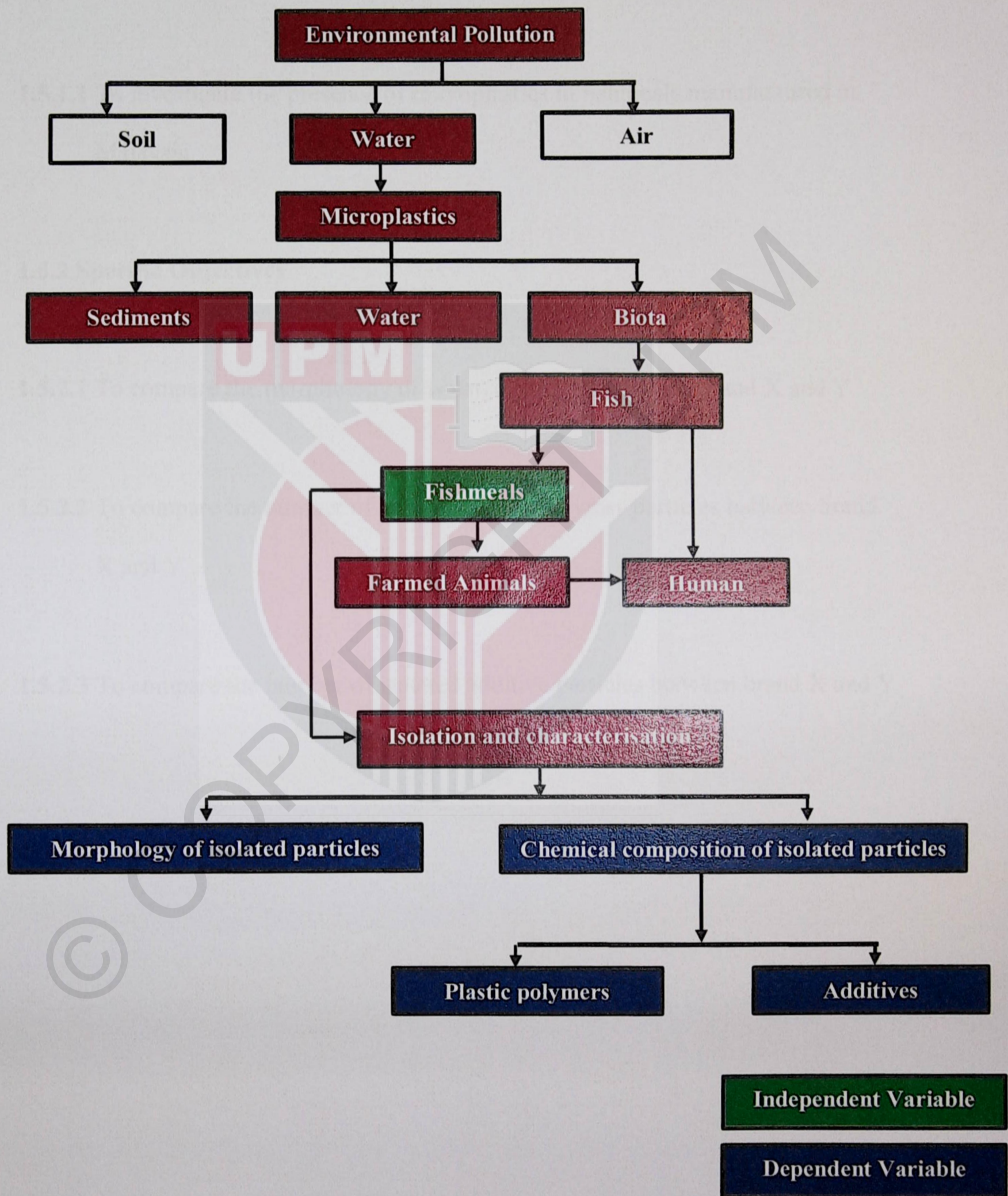


Figure 1.1: Conceptual framework on microplastics (MPs) pathways in fishmeals

## **1.5 Objectives**

### **1.5.1 General Objective**

**1.5.1.1 To investigate the presence of microplastics in fishmeals manufactured in Malaysia**

### **1.5.2 Specific Objectives**

**1.5.2.1 To compare the morphology of isolated particles between brand X and Y**

**1.5.2.2 To compare the number of isolated plastic polymer particles between brand X and Y**

**1.5.2.3 To compare the number of isolated additive particles between brand X and Y**

## **1.6 Hypothesis**

- 1.6.1** There is a significant difference on the morphology of isolated particles between brand X and Y.
- 1.6.2** There is a significant difference of the number of isolated plastic polymer particles between brand X and Y.
- 1.6.3** There is a significant difference of the number of isolated additive particles between brand X and Y.

## **CHAPTER 2**

### **LITERATURE REVIEW**

#### **2.1 Plastics as Marine Debris**

Plastics are a multipurpose material with various of importance in the industries of plastics-based products and plastics implied strong influence on the demands of manufacturing of plastics materials for the main market sectors such as agriculture (3.3%), electrical and electronic (5.8%), automotive (8.9%), building and construction (19.7%), packaging (39.9%) and others (22.4%); total demand up to 49 million tonnes (PlasticsEurope, 2016) and such demands increase the world production of plastics from 230 million tonnes (2005) to 322 million tonnes (2015) within 10 years (PlasticsEurope, 2016). Due to plastics characteristics, which were lightweight and durable; the plastics production had increased tremendously and thus resulted in increment of the amount of marine debris. Such huge production of plastics is contributing to the marine debris accumulation as a result of inhuman acts of plastics disposal onto the marine environment. Such marine debris is usually associated with man-made (anthropogenic) waste; the discard or abandoned used items such as packaging, fishing gears.

## 2.2 Microplastics and their Distribution

Before, the scientific and public attention puts an eye on the big size plastic debris. However, the presence of small plastic debris was already published in the early 1970s (Carpenter & Smith, 1972; Carpenter et al., 1972). In 2004, the term MPs have begun to spread worldwide in relation to marine debris since Thompson et al. (2004) utilise the term microscopic plastics to explain the accumulation of microscopic pieces of plastic in marine sediment and European water column.

Microplastics are distinguished into two. Primary MPs are plastics that are manufactured to be of a microscopic size such as microbeads in the skincare and cosmetic products or as air blasting media (Cole et al., 2011). Whilst secondary MPs are generated when big-sized plastics fragmented resulting from the process of environmental degradation (Duis & Coors, 2016). Sources of MPs are both marine and land based such as unintentional spillages in international and national shipping route and neighbouring countries and include spillages during handling and transfer and losses during transportation (Veerasingam et al., 2016). Besides wastewater effluent, maritime transport and tourism is also another contributor to the MPs pollution in the marine environment (Graca et al., 2017).

These plastics marine debris are accumulating in the ocean and continues to increase in numbers. As far as people concern, plastics are infamous for their non-biodegradable properties and indeed a forever nuisance to the environment. Although the size of the plastics marine debris decreases, the amount of MPs is increasing due to the fragmentation process. Plastics in the ocean are breaking down into a smaller

size due to fragmentation process under the influence of mechanical forces, light and microorganisms. Microplastics are the fragmented, unit pieces of plastics as a result of natural degradation through mechanical action (Klemchuck, 1990), with a size of 1 to 1000  $\mu\text{m}$  (Karami et al., 2016). The non-biodegradable nature of MPs made them to last longer in the environment and its buoyancy is among the causes of MPs to be dispersed far from its source of origin. In addition, MPs is capable to go over long distances with the aid from ocean currents, winds and tides (Thompson et al., 2004).

The abundance of MPs has been spotted at both terrestrial and marine environment; for example, MPs are found in Singapore's coastal water (Ng & Obbard, 2006), Northern Pacific Gyre (Rios et al., 2010), East Malaysia beaches (Noik, 2014), western tropical and sub-tropical Atlantic Ocean (Costa & Barletta, 2015) and Goa coast, India (Veerasingam et al., 2016).

### **2.2.1 Morphology of Microplastics**

With respect to their morphology, MPs are classified into 5 types based on their morphology; mainly beads (Lusher et al., 2012; Kang, Kwon & Shim, 2015), fibers (Desforges, Galbraith & Ross, 2015; Li et al., 2015; Davidson & Dudas, 2016), fragments (Li et al., 2015; Davidson & Dudas, 2016), film (Davidson & Dudas, 2016; Lusher et al., 2012) and foam (Lusher et al., 2016; Rochman et al., 2015) as shown in the Figure 2.1.



**Figure 2.1: The example of morphology of microplastics (MPs)**  
(Cole et al., 2014; Rochman et al., 2015)

The existence of MPs from raw fishes has been reported in a great extent. For example, raw fish sampled from Indonesia across 11 different species such as tilapia, oxeye scad and Indian mackerel has been found to contain MPs consist of 60% plastic fragment, 37% plastics foam and 2% plastic film in their gastrointestinal (GI) tract especially inside the gut (Rochman et al., 2015). Next, similar finding of the MPs inside the gut of raw fishes, which was planktivorous fishes in the North Pacific Central Gyre also been reported and consist of primarily fragment and film (Boerger et al., 2010).

### 2.2.2 Type of Plastic Polymers

PlasticsEurope, an Association of Plastics Manufacturers has listed 14 types of plastic, mainly bio-based plastics, biodegradable plastics, engineering plastics, elastomers, epoxy resins, expanded polystyrene, fluoropolymers, polyolefins, polystyrene, polyurethanes, polyvinyl chloride, thermoplastics and thermosets (PlasticEurope, 2017). However, there are seven common types of plastic polymers; Polyethylene Terephthalate (PET), High-Density Polyethylene (HDPE), Polyvinyl Chloride (PVC), Low-Density Polyethylene (LDPE), Polypropylene (PP),

Polystyrene (PS) and Other (Nylon (PA)), Acrylonitrile butadiene styrene (ABS), Polycarbonate (PC) and layer or multi-material mixed polymers.

The early finding of the MPs discovered PP, PE and PA in the sediment from beaches and from the estuarine and sub-tidal sediments around United Kingdom (Thompson et al., 2004). Later, Ng & Obbard (2006) had reported PE, PS, PA and PP MPs in the seawater of the Singapore's coastal marine environment whilst Noik (2015) documented the presence of the PP, PE, PET and PS along the beaches of Sarawak, Malaysia.

### **2.2.3 Type of Additives**

Plastics products influenced much in peoples' life; medicine includes artificial organs, telecommunication requires plastic smartphones and product manufacturing uses plastics packaging. Our needs and leisure relies on the combination of the plastic characteristics. These plastic products are made from the plastic polymer and mixed with a complex blend of materials known as additives. Additives ensure plastic products easier to process, add aesthetic significance, reduce costs and last long and ensure safe and sound features (BPF, 2017). There are 18 categories of additives.

Anti-counterfeiting is used to tackle counterfeiting by utilizing multilayer anti-counterfeiting technologies. Antimicrobials help to prevent deterioration of plastic material which is vulnerable to microbiological attack (BPF, 2017). Antioxidants help to prevent the oxidation process that can cause loss of impact strength, elongation and discolouration. Antistatic agents are used to prevent build-up

of static electric charge whilst biodegradable plasticisers and plasticisers are used to produce soft plastics and add flexible and degradable properties to the products (BPF, 2017).

The blowing agents are functioning to form gases in the plastics to produce a foam material. The external lubricants aid in preventing damage to the plastics during processing whilst the internal lubricants and the process aid improve processability of plastics by increasing flowability by lowering the viscosity and heat dissipation (BPF, 2017). The filler and flame retardants are functioning to improve strength and prevent ignition of flame respectively. Next are fragrances, heat stabilisers and impact modifiers which play in role to prevent decomposition of polymer during processing and enabling plastic products to absorb shock and resist impact without cracking (BPF, 2017).

Light stabilizers are used to inhibit the reactions in plastics due to chemical reaction from exposure to UV light, the pigments are used to create particular colour and reinforcements used to improve tensile strength, flexural strength and stiffness of the plastics materials (BPF, 2017).

### **2.3 Possible Risk of Microplastics Consumption for Human**

Determine the actual number of MPs are difficult to do as different plastics has different fragmentation rate and depend on UV light, temperature and mechanical action, thus the effects of MPs to human are often underestimated as there are no real

data to provide the argument besides than the number of plastics production facts and the effects to the marine organisms.

### **2.3.1 Physical Harm of Microplastics to Human**

As for MPs ingestion, most of the published studies documented MPs in the gut of marine organisms, a part of marine organisms' internal organ often unconsumed by man (Galloway, 2015). However, shellfish was excluded as mussels, clams or shrimps are eaten as a whole (Galloway, 2015). Whilst the obvious potential risk of MPs is said to be found in marine organisms, sadly there is no supporting evidence for the unintentional ingestion and uptake of MPs into human bodies through food intake and biological effect cause of the food chain (Galloway, 2015).

Microplastics have been shown to cause physical damage leading to cellular necrosis, inflammation and laceration in the gastrointestinal (GI) tract of marine organisms (Rochman et al., 2015). Imagine the entire marine population facing the similar problem; the consequences would definitely take a negative toll to consumer food safety. The questions are numerous yet there is no reliable scientific data to clear these. The problem is not only focusing on the issue of environmental pollution of plastic disposal to marine habitat; but also the consequences of MPs to the human health.

Yet, there is no supporting information yet to explain on the effect of MPs ingestion to both humans and animals due to its physical and chemical properties. It is

presumed that human who consumed the MPs indirectly from eating the animals ingested MPs; the MPs will remain inside the human bodies and accumulates.

### **2.3.2 Chemical Harm of Microplastics to Human**

Microplastics are capable to absorb, concentrate and release chemicals and naturally environmental pollutants to organisms (Endo et al., 2005; Mato et al., 2001; Ogata et al., 2009; Teuten et al., 2007). Chemical leaching from MPs could become a source of chemical into a human's body (Engler, 2012). In general, plastics contain numerous additives to produce a desirable product and enhance the properties. Thus, the chemicals, here are referring as additives are able to leach off from the plastic and emerge to the surface. Engler (2012) said, the leaching can become a chronic source of chemical into human's tissue and body' fluid despite the chemical short half-lives.

Whilst chemical is a concern to marine lives and the environment, it is impossible to estimate an actual, real cumulative risk of chronic exposure to plastics and its additives due to limited understanding and scientific information on the rate of degradation, chemical leaching into environmental matrices and food chain relation (Galloway, 2015). The mechanism of toxic substances of chemical from plastic entering the food chain still remains questionable.

Besides the amount of MPs, the accumulation of harmful chemical brought along through the MPs is a concern too. However, the effect is still a question left unanswered as there is too little publication on these. Although there is no real explanation yet; but ignoring the future effect of MPs would not hinder the problem

from occurring. However, just because a polymer degrade, does not necessarily means that resulting metabolites are not toxic themselves (Browne et al., 2015).



## 2.4 Fishmeals



**Figure 2.2 Fishmeal**

The fishmeals are originated from oil production activities and its residue was used as fertilizer but it had been dried and grounded into fishmeals for animal feeding (FAO, 1986). Fishmeals are solid product, ground, that has been obtained by removing most of the water and some or all the oil from unsold fish or fish waste that is inedible such as heads, bones and trimmings leftover and are used as animal feedstock in the diet of poultry, non-ruminants and aquaculture industries (FAO, 1986). They are made by cooking, pressing, drying and grinding the fish in machines designed for purpose. Fishmeals are preferred compare to meat meal as they provided higher protein and wide range of nutritionally valuable materials for farmed animals (FAO, 1986).

Based on recent reports from the United Nations Food and Agriculture Organization (FAO), the global per capita fish consumption is shockingly above 20 kilograms a year for the very first time. As the fisheries sector has improved tremendously, the fish left over such as fish viscera and bones are made into useful by-products such as fishmeal for aquaculture and feedstock, collagen for the cosmetic

industry and snacks too (FAO, 2016). Domestic production of fishmeals in the US was around 609 million pounds (approximately RM 3394.78 million) (Fisheries of the United States, 2015). In Malaysia, fishmeals are used as feed sources for poultry and aquaculture feeding (Wan Zahari & Wong, 2009).

The occurrence of MPs in fishmeal is as follows. As mentioned earlier, the issue of marine littering of plastics is getting out of control and the numbers are increasing each day due to the economic contribution of the plastic-based industries. The degradation of plastics into MPs in the ocean and the sea are causing problems to the food web. The MPs resemble as food (Rios et al., 2010; Teuten et al., 2007) to the fishes and ingestion of MPs causing MPs accumulation inside fish's gills and gut (Boerger et al., 2010; Lu et al., 2016; Lusher et al., 2016). Accumulation of MPs in the gut may allow possible translocation of MPs to the marine organisms tissue, which is the edible tissue (Avio et al., 2015; Devriese et al., 2015; Lusher et al., 2016). Thus, this raised concern regarding the contamination of fishmeals with MPs and MPs possible pathway into human's bodies. In addition, the chemical leaches off from MPs might produce unpredictable adverse chemical reaction to the marine organism and its eater (Barnes et al., 2009; Rocha-Santos & Duarte, 2015). There are two possible routes of intake of the MPs into a human; one is through dietary intake of fish ingested MPs and the other is the dietary intake of poultry, pigs or cultured fishes fed with contaminated fishmeals.

## **2.5 Trophic Transfer**

The global plastics production had showed a tremendous growth as they increased from 1.7 million tonnes in 1950 to 322 million tonnes in 2015 (PlasticsEurope, 2016). Since the mass production of plastics in the 1940s, the plastics contamination had become a concern because plastics had made their way to the ocean and sea. It is estimated that approximately 5.25 trillion plastic particles are floating on the surface of the sea (Environmental Health Perspectives, 2016). In addition, these plastics are able to break down to a smaller size via mechanical action, sun and organism and thus, raising the issue on the MPs contamination in the marine environment. Trophic transfer from preys could be a pathway to larger individuals.

In the case of fishmeals, the trophic transfer may occur as following. Abundance of these MPs in the ocean making the fishes to mistaken the MPs as food due to the similarity of MPs to the prey in term of colour and shape and presence of microplastics can be found in the gut and gill of fishes (Foekema et al., 2013; Lusher, McHugh, & Thompson, 2013; Rochman et al., 2015). Thus, when the fishes were caught for fishing industries, these MPs-ingested fishes were sent to be sold at market whilst their fish waste such as their head, bone, gut and gill were sent to the factories to be processed into fishmeals, a feedstock for poultry, non-ruminants or aquaculture farming.

Fishmeals are used extensively as feedstock because it provided protein sources to the poultry, non-ruminants and aquaculture animals, hence indirectly transfer the MPs into these animals as they feed upon the fishmeals that contained

MPs. As everyone aware, these farmed animals are the food sources that often consumed by human. As a result, human may indirectly consumed MPs by eating these 'contaminated' farmed animals. Hence, upon the consumption by larger species, the MPs may accumulated in their tissue.

Another example of the absorption and ingestion of MPs by organisms from the primary trophic level such as zooplankton also could make up it way to the food chain. Yet, there were only a few of them. One of them is the study by Eriksson & Burton (2003) where they concluded that MPs found in the fur seal actually ingested by its prey, the plankton feeding Mycophiids. Similarly, in Farrell & Nelson (2013), 0.5  $\mu\text{m}$  PS transit from mussel to crab by trophic transfer; where PS were found in the tissue samples from the stomach, hepatopancreas, ovary and gills of crab. In regard to the trophic transfer, this emerging issue of MPs raised concern on the possibility of MPs to be transferred to human's tissue and further studies are very recommended to heighten our understanding on mechanism of trophic transfer of MPs to either human or other organism.

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1. Samples

Two types of fishmeals were purchased from the Malaysian fishmeals manufacturer, one was 55% of protein content (brand X) and the other was 60% protein content (brand Y). According to the manufacturers, both fishmeal X and Y were made up from non-commercial, small fishes mainly mackerel from the South China Sea. Brand X had less protein content compared to brand Y because brand X was made up from fish waste from fish that was meant to produce surimi where they only took fish edible part only. So the leftover or fish waste such as fish gut, fish scales, fish head and fishbone were taken by manufacturers to produce fishmeals. Meanwhile, brand Y which has higher protein composition was made up of whole body of fish (mixture of flesh and gastrointestinal tract). Each of the fishmeal was replicated with n=11 and total samples up to 22. The samples were stored in the refrigerator at 4°C in closed containers until the experiment was conducted.

## **3.2 Chemical and Materials**

### **3.2.1 Chemicals**

Sodium iodide (NaI), potassium hydroxide (KOH) and ethanol 95% were supplied by R&M Chemicals (UK).

### **3.2.2 Filter membranes**

Filter membranes No. 540 (hardened ashless, pore size 8  $\mu\text{m}$ ) and GF/D microfiber filter papers (pore size 2.7  $\mu\text{m}$ ) were purchased from Whatman and the 149  $\mu\text{m}$  filter membranes were supplied by Spectrum Laboratories (USA).

### **3.2.3 Instruments or Equipment**

The following instruments and equipment were used during the study. 250 mL DURAN glass bottles were supplied by Schott (Germany). The vacuum pump (Gast vacuum pump, DOA-P504-BN, USA) was connected to filter funnel manifold (Pall Corporation, USA). Microscope (Motiz SMZ-140 Stereomicroscope) was supplied by Motic (China). Camera apparatus used was AxioCam, ERc 5S (Germany). Micro-Raman spectroscopy used was Horiba LabRam HR Evolution.

## **3.3 Chemical and Solution Preparation**

### **3.3.1 Potassium Hydroxide (KOH) Preparation**

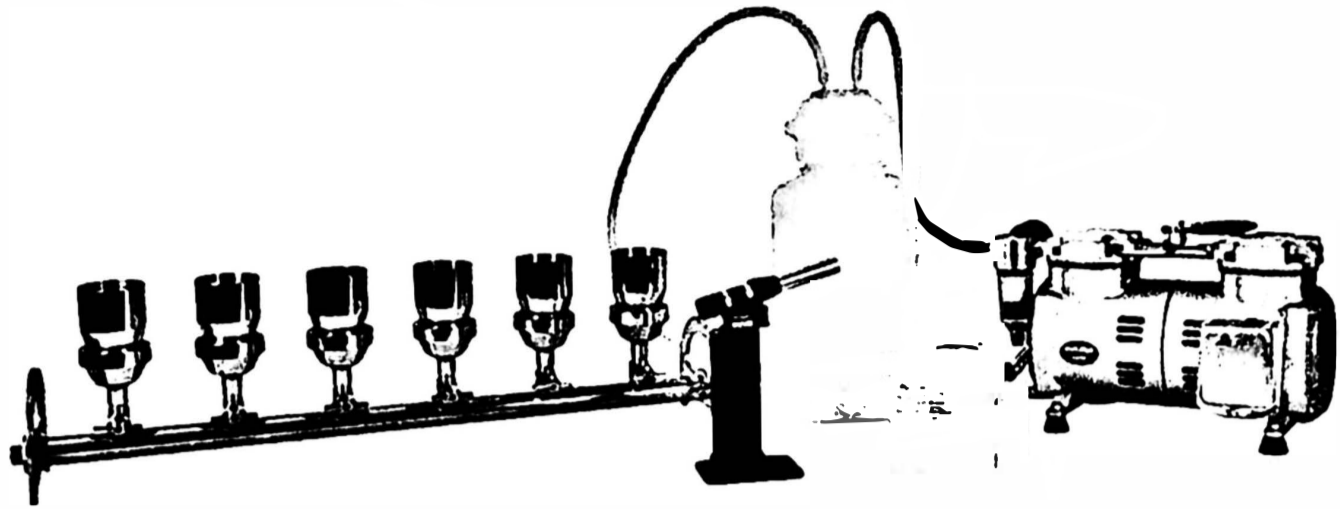
Two hundred g of KOH (R&M Chemicals, UK) was weighed and poured into a 2 L bottle. The KOH solution was prepared in the ratio of 1:10 by adding in 2 L of distilled water into the bottle. The bottle was shaken to mix the solution.

### **3.3.2 Sodium Iodide (NaI) Preparation**

A 4.4 M NaI (R&M Chemicals, UK) solution was prepared by weighing 65.95 g of NaI and put into a 50 mL beaker and gradually diluted using distilled water. Next, the solution was poured into the conical flask and distilled water was added in until it reached 100 mL and swirled to mix the solution evenly.

### **3.4 Digestion and Filtration**

Twenty g of fishmeal were weighed, put into a 250 mL DURAN glass bottle (Schott, Germany) to be digested using 10% KOH and incubated in an oven at 40°C for 72 h with 1:10 ratio (20 g fishmeal:200 mL 10% KOH). The digestion process was considered as complete if it appears clear and no visible fishmeals material remains. Filtration of digested sample was conducted via the multiple vacuum filtration (Figure 3.1) using filter paper pore size 149  $\mu\text{m}$  (Spectrum Laboratories, USA) and filter paper Whatman No.540 (hardened ashless, pore size 8 $\mu\text{m}$ ) according to the order. The first filter paper (149  $\mu\text{m}$  filter paper) was soaked with 15 mL 4.4 M NaI and placed into the sonicator for 10 min. The first filter paper was removed onto petri dish and the sample was centrifuged at 500 rpm for 1 min. The supernatant was filtered using 8  $\mu\text{m}$  filter paper (Karami et al., 2016).



**Figure 3.1: Multiple vacuum filtration**

### **3.5 Identification and Characterisation of Microplastics**

#### **3.5.1 Visual Characterisation**

The 8  $\mu\text{m}$  filter paper was observed under Motiz SMZ-140 stereomicroscope (Motic, China) to identify and isolate the MP-like particles according to their morphologies and colour. The carbon-based materials, plant tissues and remains of invertebrate exoskeletons can be found on the filter paper as these particles cannot be excluded through density separation. The remains of invertebrate were mostly yellow, light brown or black in colour whilst the plant-based items came in dark brown or black in colour. Extra-precaution step was taken during brown and black particles sampling due to their similar look to non-plastic items. Microplastic-like particles were categorized into bead (rounded particles), fiber/filament (thin, straight and often cylindrical particles), film (thin plane of filmsy particles), foam (lightweight particles with spongy texture) and fragment (jagged and irregular shape particles with an uneven surface) (Free et al., 2014). The representatives MPs pictures were taken using a camera apparatus (AxioCam, ERc 5S, Germany) attached to the

stereomicroscope. The largest cross-section of the isolated particles was examined using ImageJ software.

### 3.5.2 Micro-Raman Spectroscopy

The isolated particles were put onto microscope slide and kept inside a clean and dried petri dish for further identification for their chemical composition by using the micro-Raman spectroscopy (Figure 3.2) equipped with a Single Mode Open Beam laser Diode (Innovative Photonic Solutions). The sample particle was shot under the beam of laser with wavelength of 785 nm to obtain the data peaks. All acquired spectrums then compared with the online polymers spectral library for the plastics identification.



**Figure 3.2: Micro-Raman spectroscopy**

### 3.6 Statistical Analysis

Before the analyses, data had been checked for normality. Data were log-transformed when required to meet the assumptions of normality (Shapiro-Wilks

test). The data were analysed with IBM SPSS Statistics (V. 22). The parametric test (Independent t-test) was used to conduct statistical analyses of the normal data whilst the non-parametric test (Mann-Whitney U test) was used to conduct the statistical analyses of non-normal data. The results with  $p < 0.05$  were considered statistically significant.

### **3.7 Procedural Blank and Contamination Prevention**

One procedural blank for each fishmeal brand was performed to correct the potential procedural contamination. The experiment was conducted in the horizontal laminar cabinet (Model AHC-4A1-ESCO) to prevent contamination of the samples. Before experiment was conducted, the working area was cleaned. The surface area of the horizontal laminar cabinet was wiped thoroughly using 70% ethanol solution which was put onto tissue. The glasswares were washed once with a commercial dishwashing liquid, rinsed with ultra-purified distilled water and finally with ethanol and dried in an oven. Cotton laboratory coat, nitrile gloves and face mask was worn during the entire experiment. To avoid contamination, ethanol was filtered with 2.7  $\mu\text{m}$  glass fiber filter paper (Whatman).

### **3.8 Ethics Statement**

The research study is exempted from the Ethics Committee of Universiti Putra Malaysia as there was no human subject included.

## CHAPTER 4

### RESULTS

#### 4.1 Isolated Particles and their Morphology

There is a significant difference on the morphology of isolated particles between brand X and Y as the total isolated particles (IPs) (film, fragment and filament) has a value of  $p < 0.05$  in Independent t-test (Table 4.1). Among the three morphologies, there is a significant difference of fragment between both fishmeals compared to filament and film.

**Table 4.1: Mean comparison of isolated particles morphology between brand X and Y (n=11)**

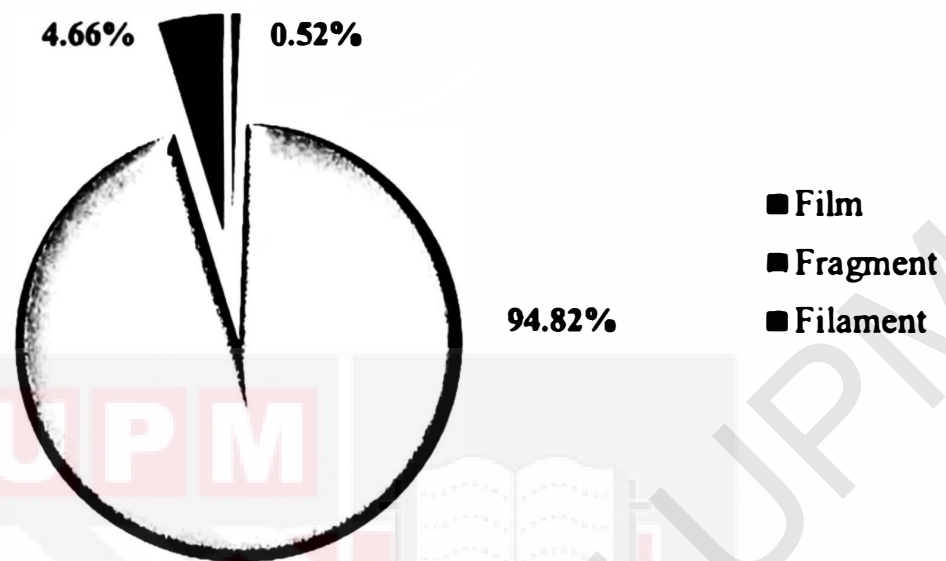
Variable	Mean (SD)		Mean difference (95%)	T-statistics (df)	p-value
	X	Y			
Fragment	1.20 (0.21)	0.60 (0.40)	0.601 (0.30,0.89)	4.37 (20)	*<0.01
Filament	0.20 (0.21)	0.10 (0.15)	0.098 (-0.06,0.26)	1.22 (20)	0.236
Total IPs	1.22 (0.22)	0.65 (0.36)	0.565 (0.29,0.83)	4.37 (20)	*<0.01

Independent t-test

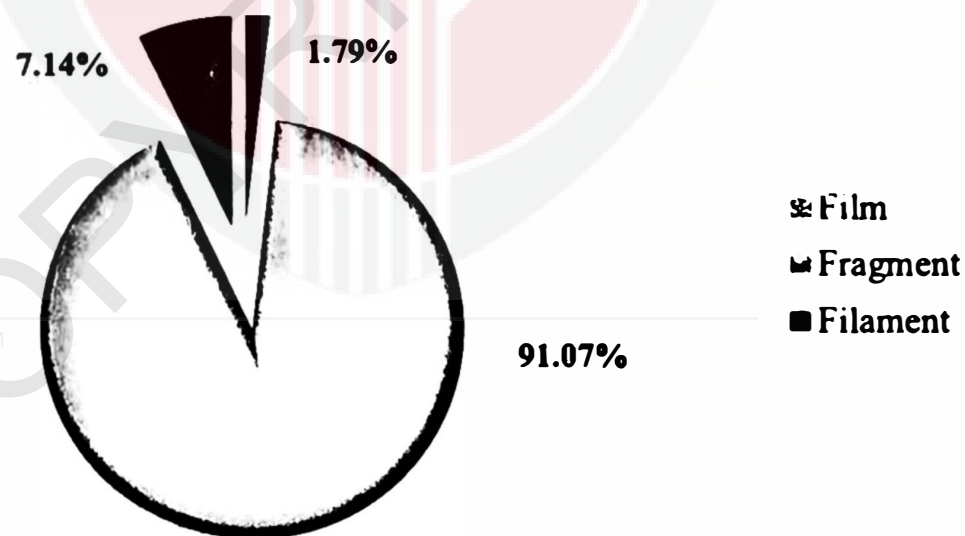
\* $p < 0.05$  is significant

As shown in the Figure 4.1 and Figure 4.2, fragment is the predominant type of particle morphology found in both fishmeals, where, brand X (94.82%) has a greater percentage of fragment than brand Y (91.07%). However, brand Y has higher

percentage of filament (7.14%) and film (1.79%) compared to brand X, which is 4.66% of filament and 0.52% of film.

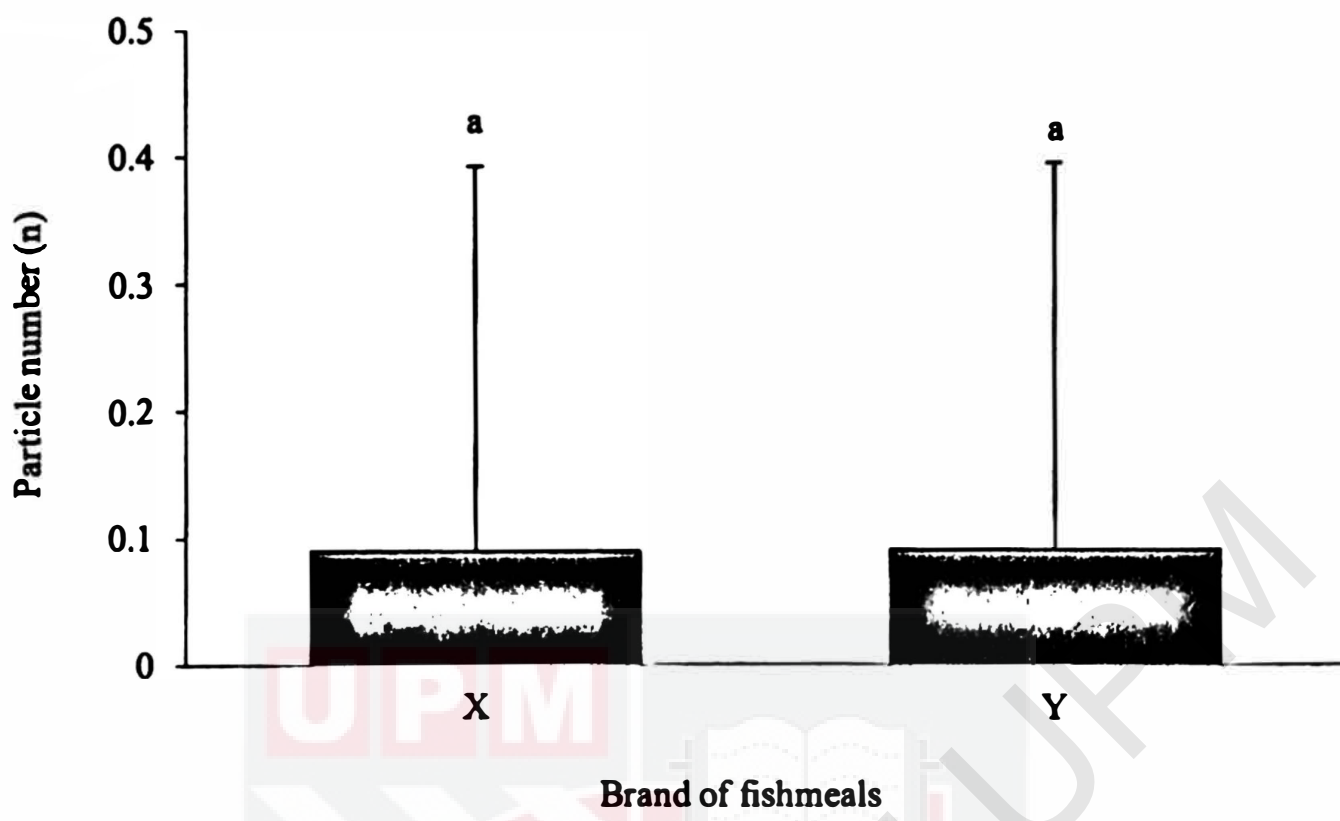


**Figure 4.1: The percentage of morphology of isolated particles in brand X (n=11)**

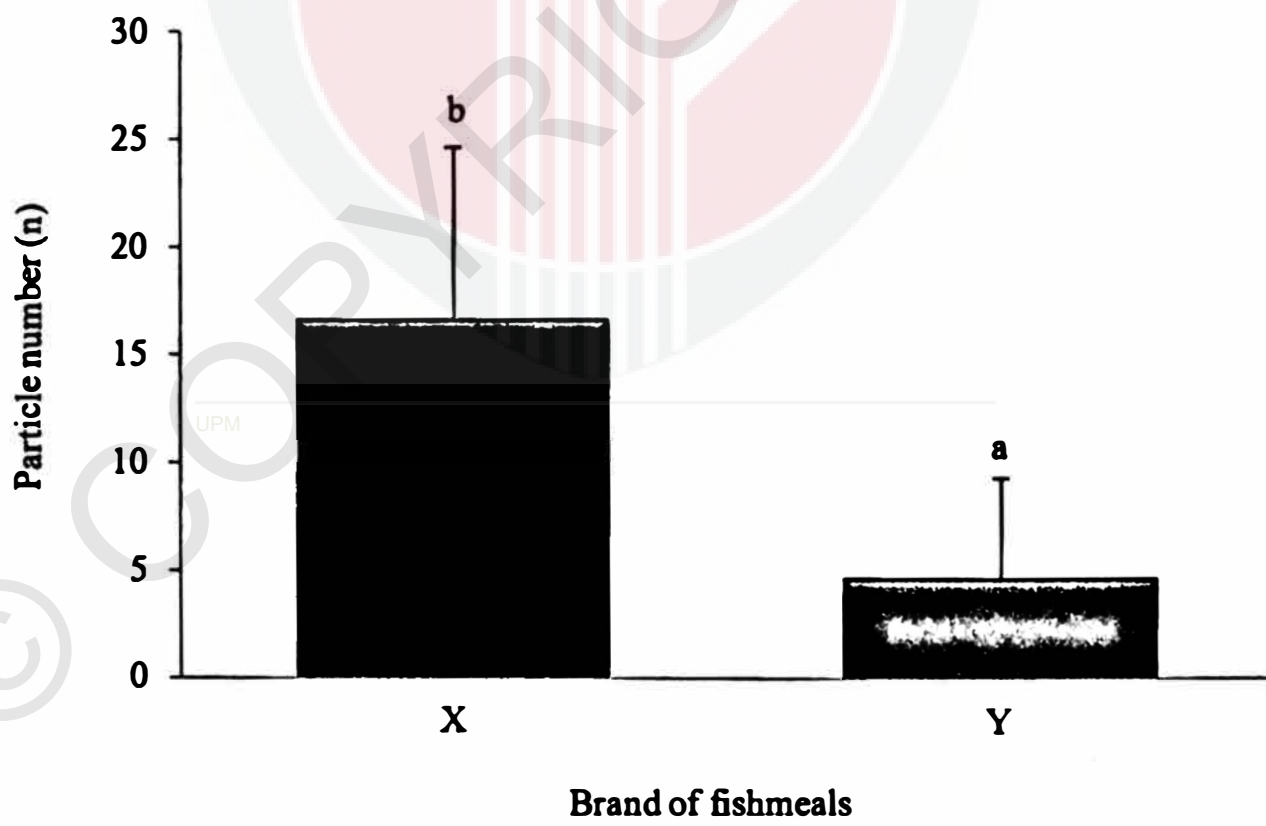


**Figure 4.2: The percentage of morphology of isolated particles in brand Y (n=11)**

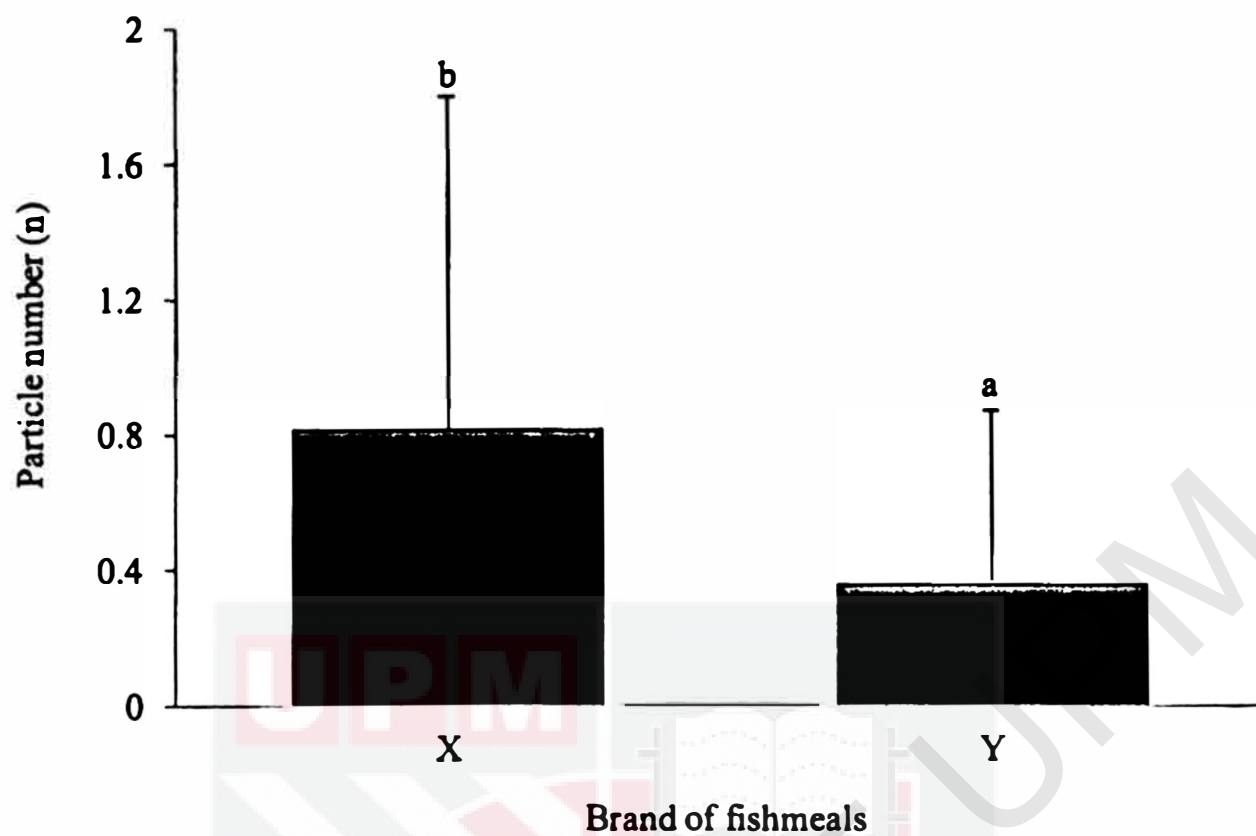
There is no significant difference of mean of film between brand X and Y (Figure 4.3) because both brand consisted of only one film particle only each with mean of 0.09 whilst there is a significant difference of mean of fragment and filament in both X and Y (Figure 4.4 and Figure 4.5).



**Figure 4.3: Mean film in brand X and Y (n=11)**



**Figure 4.4: Mean fragment in brand X and Y (n=11)**



**Figure 4.5: Mean filament in brand X and Y (n=11)**

#### 4.2 Type of Plastic Polymers

Table 4.2 presented that there is a significant difference of the total plastic polymers (PE, PP and PET) with  $p < 0.05$  between the fishmeals. Both PE and PET showed significant difference in both fishmeals compared to PP.

**Table 4.2: Mean comparison of plastic polymers between brand X and Y (n=11)**

Variable	Mean (SD)		Mean difference (95%)	T-statistics (df)	p-value
	X	Y			
<b>Polypropylene</b>	0.39 (0.26)	0.24 (0.28)	0.148 (-0.09,0.39)	1.27 (20)	0.218
<b>Total plastic polymers</b>	1.04 (0.17)	0.63 (0.35)	0.414 (0.16,0.66)	3.44 (20)	*0.003

Independent t-test

\* $p < 0.05$  is significant

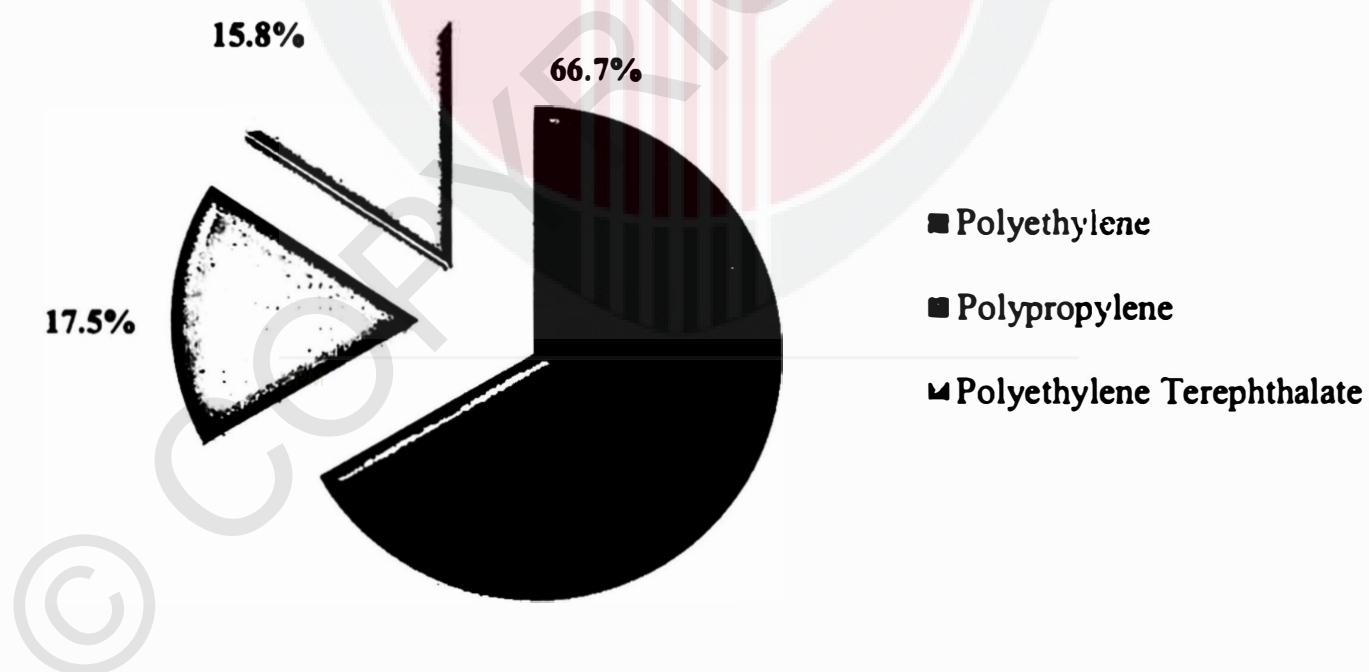
**Table 4.3: Median comparison of plastic polymers between brand X and Y (n=11)**

Variable	X	Y	z	p-value
	Median (IQR)			
<b>Polyethylene Terephthalate</b>	0.47 (0.48)	0.00 (0.48)	-3.36	*<0.01
<b>Polyethylene</b>	0.30 (0.35)	0.00 (0.35)	-3.37	*<0.01

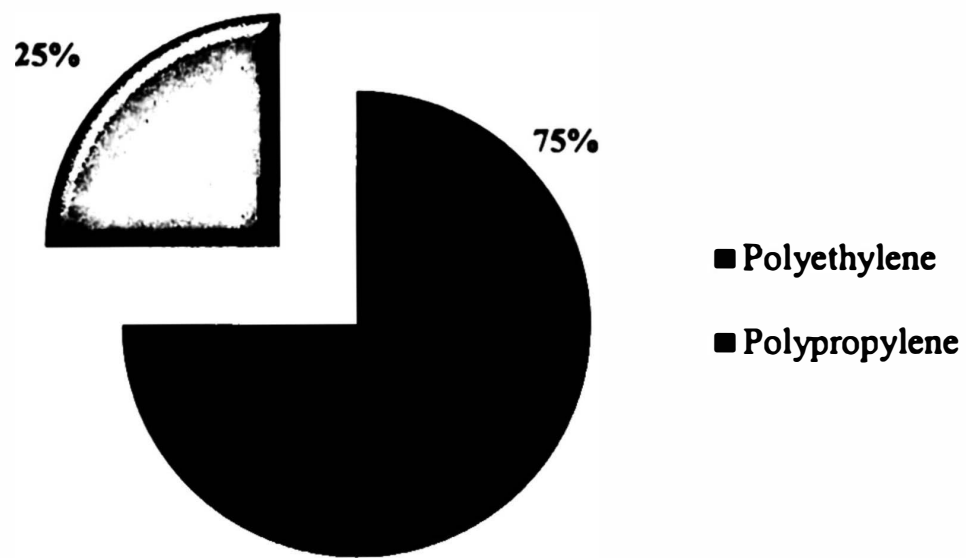
Mann-Whitney U Test

\* $p < 0.05$  is significant

Polyethylene is predominant plastic polymer in both brand X (66.7%) and brand Y (75%). In addition, brand Y has 0% of PET whilst brand X has 15.8% (Figure 4.6 and Figure 4.7). However, brand Y has higher percentage of PE (75%) and PP (25%) compared to brand X, 66.7% of PE and 17.5% PP.

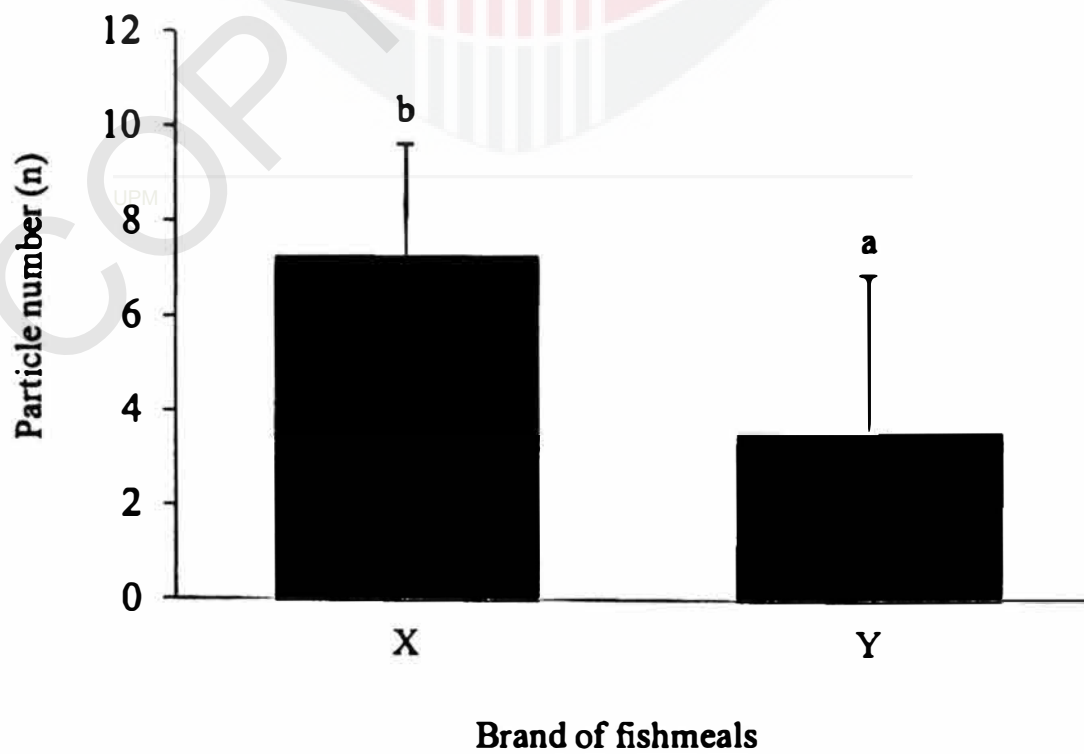


**Figure 4.6: The percentage of plastic polymers in brand X (n=11)**

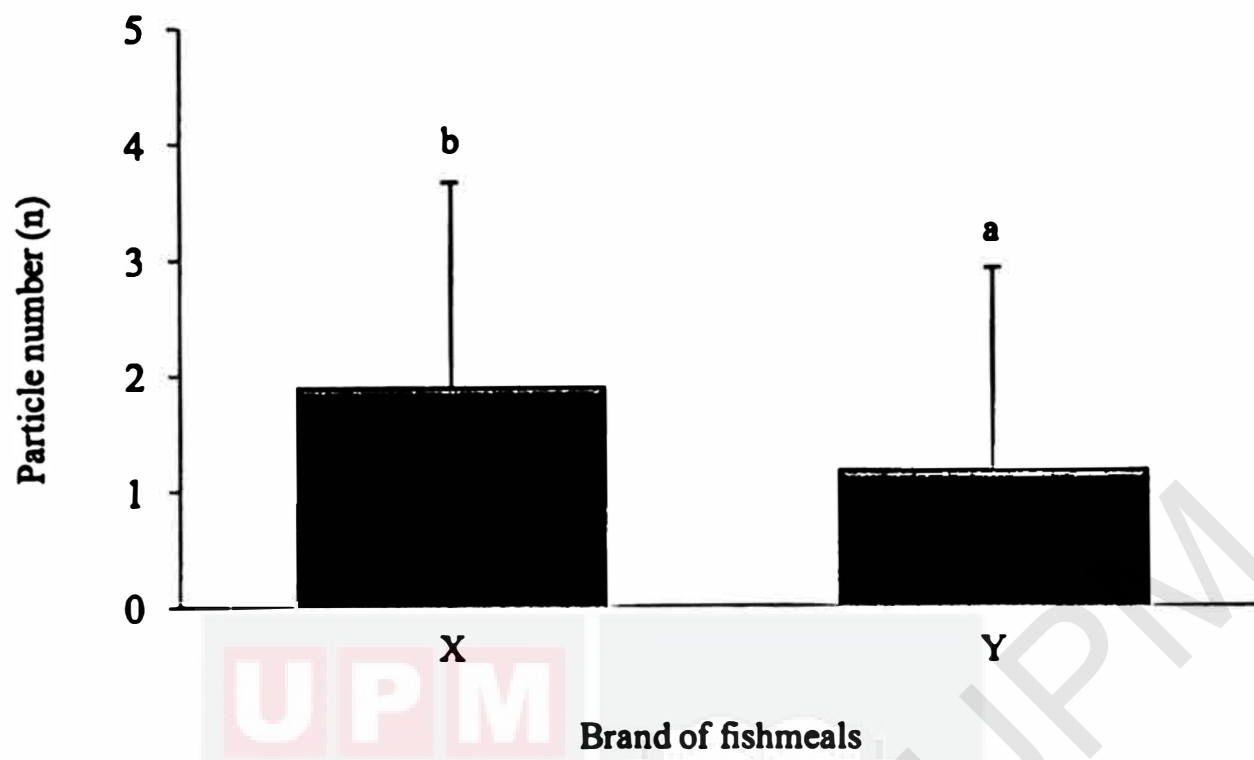


**Figure 4.7: The percentage of plastic polymers in brand Y (n=11)**

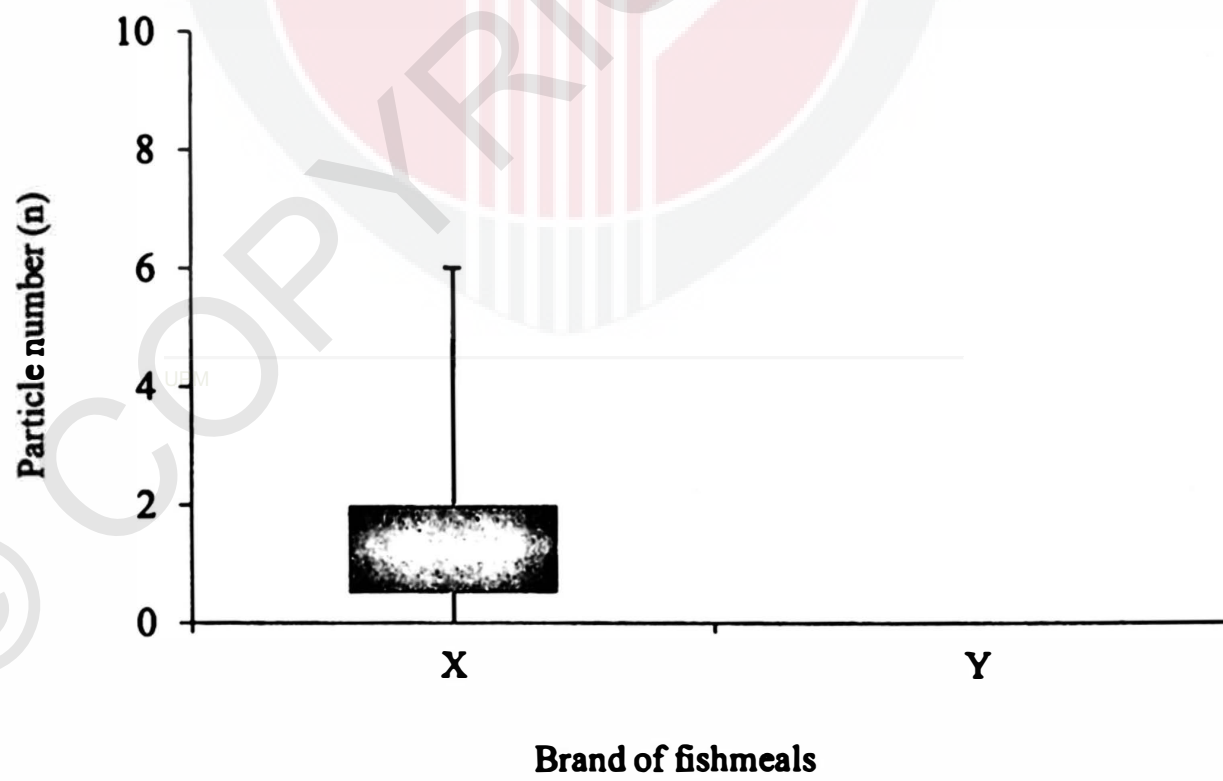
There is no significant difference on mean of PP between two fishmeals (Figure 4.9) whilst there is significant difference of mean and median of PE and PET in both fishmeals (Figure 4.8 and Figure 4.10).



**Figure 4.8: Mean polyethylene in brand X and Y (n=11)**



**Figure 4.9: Mean polypropylene in brand X and Y (n=11)**



**Figure 4.10: Median polyethylene terephthalene in brand X and Y (n=11)**

### 4.3 Type of Additives

All three; total additive, phthalocyanine and hostasol green have a significant difference in both fishmeals with  $p < 0.01$ .

**Table 4.4: Mean comparison of additives between brand X and Y (n=11)**

Variable	Mean (SD)		Mean difference (95%)	T-statistics (df)	p-value
	X	Y			
<b>Phthalocyanine</b>	0.61 (0.36)	0.08 (0.14)	0.530 (0.27,0.78)	4.45 (20)	*<0.01
<b>Total additive</b>	0.72 (0.44)	0.08 (0.14)	0.645 (0.34,0.94)	4.62 (20)	*<0.01

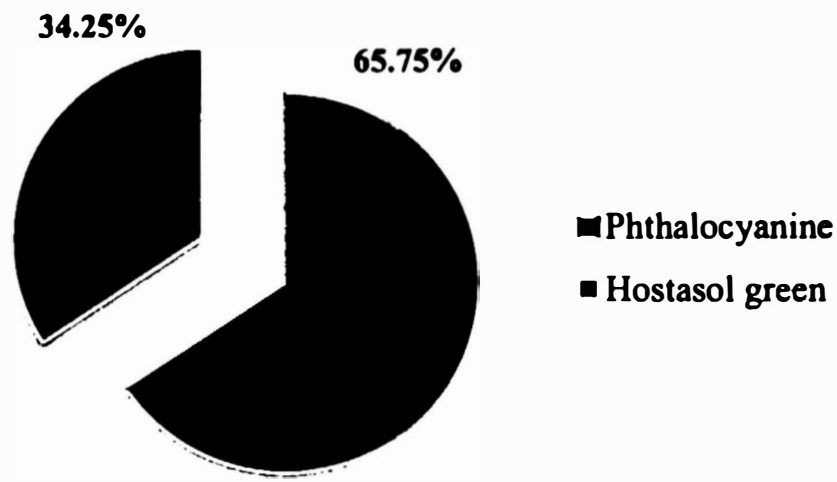
Independent t-test  
\* $p < 0.05$  is significant

**Table 4.5: Median comparison of additives between brand X and Y (n=11)**

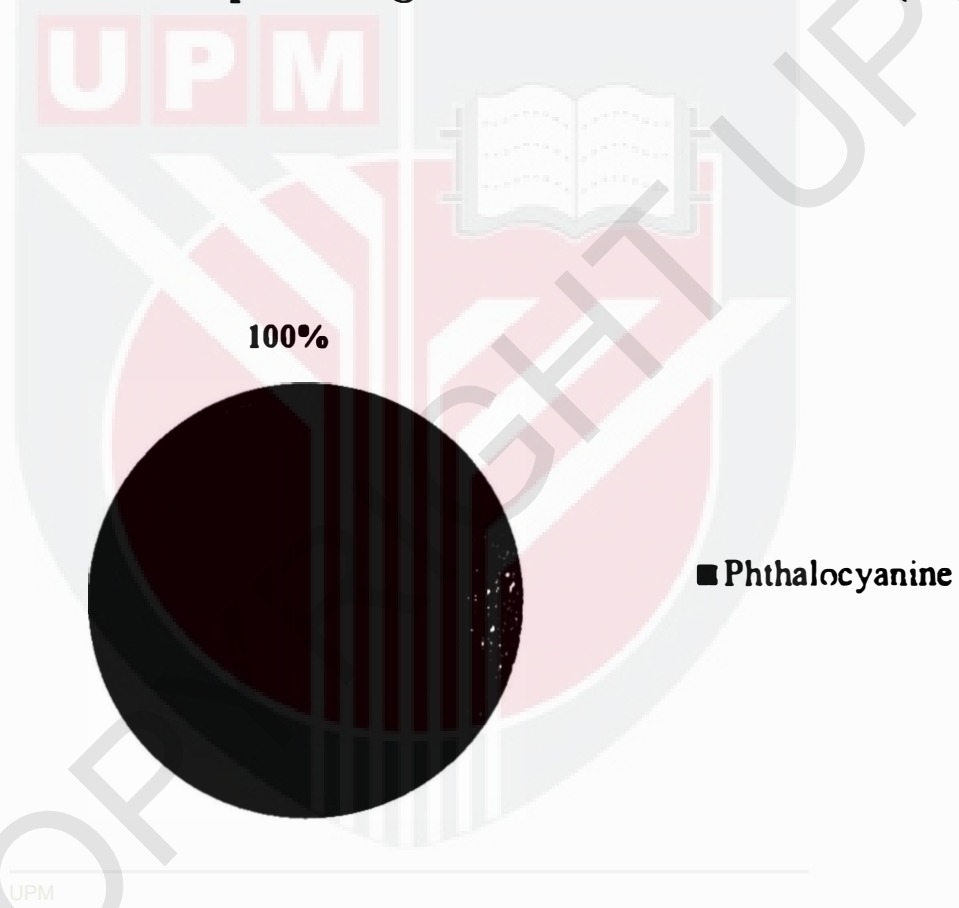
Variable	X	Y	z	p-value
	Median (IQR)			
<b>Hostasol green</b>	0.30 (0.35)	0.00 (0.35)	-3.362	*<0.01

Mann-Whitney U Test  
\* $p < 0.05$  is significant

Phthalocyanine is present the most in both fishmeals with 65.75% (n=48) in X and 100% (n=3) in brand Y. However, brand Y contains no hostasol green compared to brand X that has 35.25% (n=25) hostasol green.

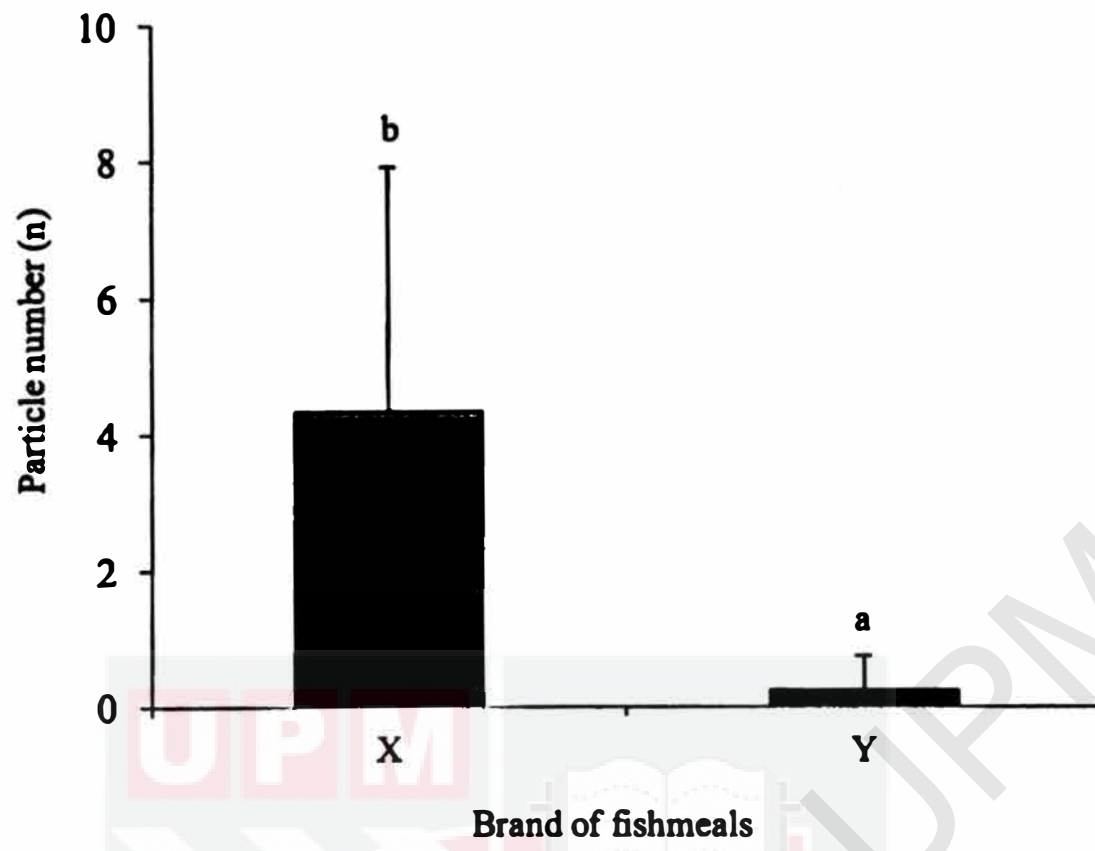


**Figure 4.11: The percentage of additives in brand X (n=11)**

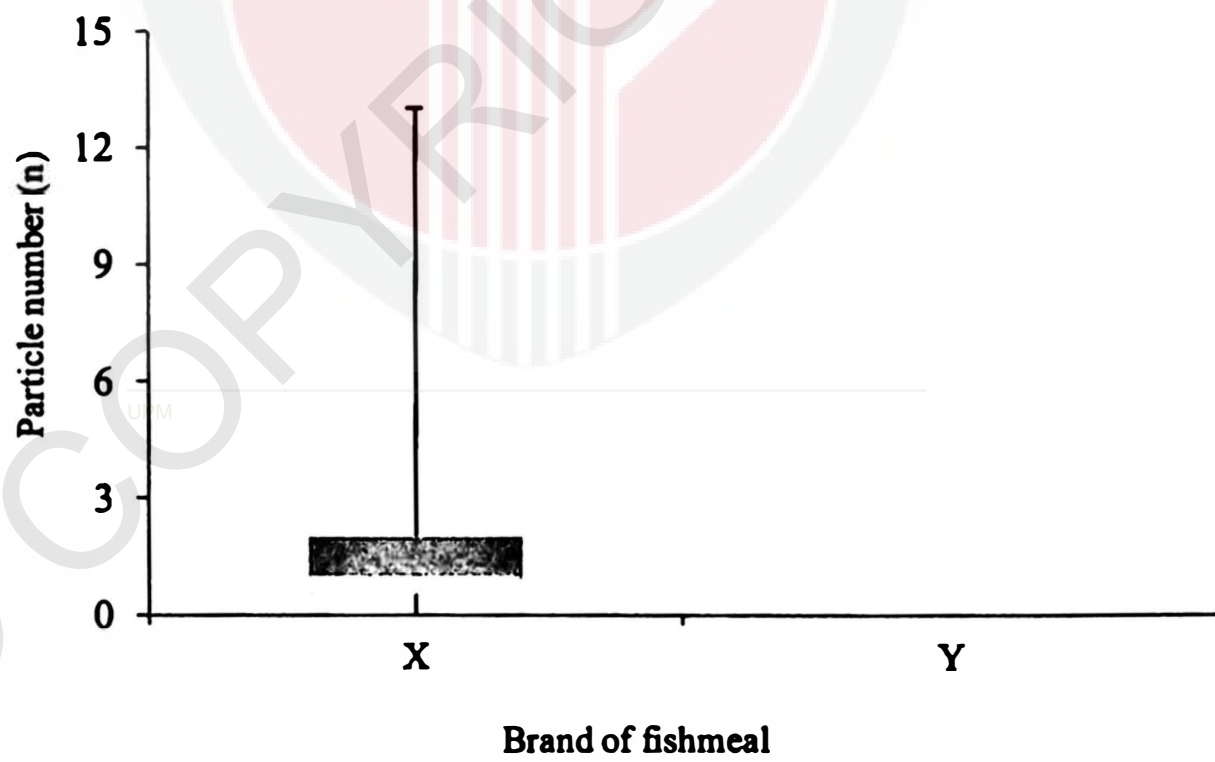


**Figure 4.12: The percentage of additives in brand Y (n=11)**

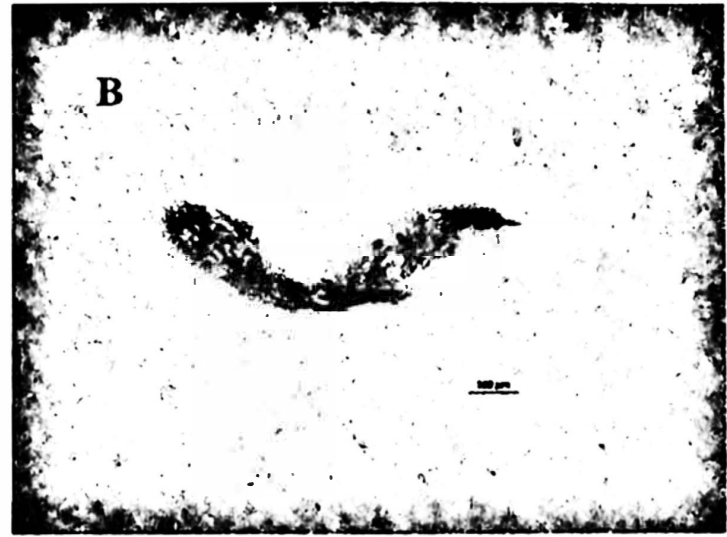
Both phthalocyanine and hostasol green showed a significant difference of mean and median in both fishmeals.



**Figure 4.13: Mean phthalocyanine in brand X and Y (n=11)**



**Figure 4.14: Median hostasol green in brand X and Y (n=11)**



**Figure 4.15: Representative microplastics as visualized using stereomicroscope (A. polyethylene fragment, B. polyethylene terephthalate + phthalocyanine fragment, C. polyethylene fragment, D. polypropylene + hostasol green fragment)**

## **CHAPTER 5**

### **DISCUSSION**

#### **5.1 Isolated Particles and their Morphology**

In total, brand X (n=193) has more number of isolated particles compared to brand Y (n=56). It is possibly due to the difference of the fishmeal's main raw materials. According to the manufacturer, brand X is made up of the uneaten part of fish or also known as fish waste such as head, gill and bone and gut whilst brand Y is made up of a whole fish. In line with the previous studies, MPs can be found a lot in the gastrointestinal (GI) tract of the organisms such as gut and gill. For example, MPs is shown to accumulated in the gut cavity of birds, fish and polychaete worms (Browne et al., 2008; Lusher, McHugh & Thompson, 2013). Although brand Y was supposed to yield similar outcome as brand X because brand Y also contained fish waste; however, according to manufacturer, in comparison to 1 kg proportion of each fishmeal, brand X has more fish waste. That could justify the difference in number of MPs in each fishmeal.

Despite the difference in number of isolated particles, the fact is both fishmeals contained MPs. One of the reasons is due to the fish it made of, which was mackerel; described as feeding by two methods. First is the pelagic-neritic feeder, mackerel that fed at the pelagic zone, an area that neither nearby the shore nor near the seafloor surface. The feeding behaviour of the mackerel is described as filtering

out the smaller pelagic organisms mainly plankton organisms (FishBase, 2017) and MPs ingestion by plankton had been well-documented in a number of studies (Cole et al., 2013; Desforges et al., 2015).

However, there were some MPs found in the study are too large to be ingested by planktonic organisms. The larger MPs were found probably because mackerel eat all kinds of small fish (Fishbase, 2017). The adults fed upon various invertebrates such as copepods (Desforges et al., 2015; Kang et al., 2015), shrimps (Devriese et al., 2015), crab (Farrell & Nelson, 2013) and small herring (Foekema et al., 2013; ochman et al., 2015) and such invertebrates recorded presence of MPs.

The basic characteristic of the MPs (lightweight and floatable) was suggested to be the contributing factor to the mackerel's ingestion of MPs. The MPs are foreign matters in the marine environment and marine organisms who are not familiar with them will mistook those MPs as foods (Barasarathi et al., 2011; Noik, 2015; Rios et al., 2010). Furthermore, the colour of MPs also an another ingestion factor as most plastics are dark-coloured and it projected the same colour as the preys (Lusher, McHugh & Thompson, 2013).

Film, fragment and filament are the common morphology found in both brand X and Y, where fragment had the highest frequency. There is no supporting data to explain the prevalence of these morphologies. However, it may be due to the fragmentation process via mechanical action much a less contributed to this irregular shape and uneven surface.

In contrast of the high number of the MPs found in fishmeals, there are chances of loss of MPs during the filtration process. As for the first filtration, filter paper size used was 149  $\mu\text{m}$  and it is possible for smaller MPs to pass through the filter paper. Yet, due to technical limitation, reduction of the size of filter paper cannot be done as it will caused blockage problem during the filtration process and may induce a greater loss of the MPs due to technical problem and where supposedly there is more number of MPs to be isolated.

## 5.2 Type of Plastic Polymers

Polyethylene (n=119) and polypropylene (n=34) are the top two plastic polymers found in the fishmeals and it is in line with the fact as the most used plastics in the world (PlasticsEurope, 2016). Polyethylene is often used to produce toys, bottles shampoo, reusable bags and food packaging film whilst PP is used in the production of the food packaging, automotive parts and snack and sweet wrappers. These mentioned items are the common used plastics in the world (PlasticsEurope, 2016).

Density separation is used to extract microplastics from bulk sediment samples and usually, plastic particles have lower weight than sediments (Duis & Coors, 2016). Thus, when fishmeal samples are mixed with salt solution like NaI, the fishmeals settled at the bottom whilst MPs can be collected on the surface. Meanwhile, the density of both PE and PP is lower than that seawater and water, which are 0.89 to 0.98  $\text{g}/\text{cm}^3$  and 0.83 to 0.92  $\text{g}/\text{cm}^3$  respectively and plastic polymers with a lower density were predominantly found in pelagic fish species (Duis & Coors,

2016). For instance, the low-density MPs are ingested by pelagic filter feeders, without discriminated the food (Wright, Thompson & Galloway, 2013). They will capture food without further selection (Moore, 2008).

It is no surprise as the presence of these two plastic polymers had been reported countless times in the sediment or water column; although the seawater movements in the ocean and the wind had contributed to the dispersion of the MPs to an unimaginable distance (Kershaw et al., 2011; Ng & Obbard, 2006; Noik, 2015; Thompson et al., 2004).

### **5.3 Type of Additives**

The isolated particles, phthalocyanine and hostasol green were identified as pigments as the strong Raman signal of these pigments inhibited identification of plastic polymers (Karami et al., 2017). Phthalocyanine and hostasol green are two additives that were found in the fishmeals. The additives may not necessary be MPs but the additives may appear to be the colorant pigment, an insoluble compound that is used widely in the production of the paints, ceramics and plastics (Gürses et al., 2016). According to Karami et al., (2017), pigmented particles had an anthropogenic origin, however, it is unsure whether they were MPs.

The first additive, phthalocyanine are used as blue or green inks pigment that features extreme light fastness and known to have resistance to solvents, acid and alkali (Bott, 2007; Ventura & Mazuca, 2014). The presence of phthalocyanine also mentioned in the bivalves consumed for human (Van Cauwenberghe & Janssen, 2014). In addition, phthalocyanine is a synthetic pigment, which used a lot in the

plastic industries (Pigments and Additives Division - Clariant International Ltd, 2007).

The second additive, hostasol green, however, has not been recorded its presence in any studies; there is no finding of hostasol green in marine organisms of any studies. In addition, the available studies also did not aim to find the presence of the plastic additives, thus make it difficult to discover either the effect it caused or its prevalence in the marine environments. Additives caused difficulties in plastic identification as they alter the polymer spectra and hinder comparisons with the reference library and unavailability of comprehensive spectra library to identify mixed samples (Karami et al., 2017).

#### **5.4 Possibility of Microplastics Translocation into Tissue**

There are several reports that documented on the translocation of the MPs from the gut and gill of fish and other invertebrates to the hepatic tissue via circulatory system. This raised a concern on the possibility of translocation of these MPs into the edible tissue in the poultry or farmed fish upon fed with contaminated fishmeals. Few studies had been conducted to observe the exposure of MPs in marine organism tissues. In Farrell & Nelson (2013), 0.5  $\mu\text{m}$  PS transit from mussel to crab by trophic transfer; where PS were found in the tissue samples from the stomach, hepatopancreas, ovary and gills of crab. Next is translocation to hemolymph and blood cell of mussel after ingestion of MPs (Browne et al., 2008; Von Moos et al., 2012).

Although filter-feeding organisms are unlikely to break down, digest or absorb plastics due to lack availability of enzymatic pathways, however, they may pass through cell membranes and become incorporated into body tissue following ingestion and translocated across the gut epithelial lining into the circulatory system; though the precise uptake mechanism remains questionable (Wright, Thompson, et al., 2013).

However, the following laboratory studies had showed the translocation of MPs from the gut to the circulatory system and various tissues and cells in human and other mammals and its system. In Grafmueller et al. (2015), ex vivo study using human placenta showed that particle up to 240 nm were taken up by the placenta. Besides, absorption of PE particles was taken up in lymph and circulatory system from gastrointestinal (GI) tract of human (Hussain et al., 2001). However, no finding had yet showed any translocation of MPs in human from ingestion of poultry and fish. Based on such finding, there are chances of MPs to be translocated into the edible tissues of farmed animals upon feeding with contaminated fishmeals. Upon ingestion of such contaminated protein sources, human are vulnerable to health-risk adverse effects.

Issue of MPs are related to the marine environment because that's where the fragmentation occurs due to mechanical, chemical and biological forces; that's justified the reason why majority of the studies are focused on marine organism. Based on the previous studies, most of the MPs were only documented on marine organisms such as fishes, mussels, shrimps and bivalves. Despite the possibility of translocation of MPs to human or other organisms such as poultry or non-ruminants,

there were less studies reported on MPs found in other organism but none were reported in human. For example, MPs can be found in stranded Northern Fulmars and Sooty Shearwaters, a species of birds on the Oregon and Washington beaches (Terepocki et al., 2017). Another example is the ingestion of marine debris by the seabirds and the study found several type of MPs in stomach and gizzard of different type of seabirds such as *Phalaropus lobatus*, *Oceanites oceanicus* and *Puffinus gravis* (Moser & Lee, 1992; Franeker & Law, 2015).

### **5.5 Health Effects of Microplastics to Human**

Microplastics may not affect much in term of bodily injury because of its unit size, but the studies indicated that the pollutants (chemicals) adsorbed or absorbed by MPs either from manufacturer or from the polluted environment can cause harm to human (Nerland et al., 2014). The number and types of chemicals that have been reported sorbed to plastic pellets, particles and debris is relatively low considering the number of plastic additives used and contaminants present in the marine environment, however this may be due to the limited number of studies performed to date and the limited focus of the analyses performed (Nerland et al., 2014). Most of the chemicals identified and quantified in plastic debris are relatively hydrophobic and many of them have been classified as persistent chemicals (Cole et al., 2011; Nerland et al., 2014; Teuten et al., 2007).

Most of the concentrations reported have been measured in particles in the millimeter size range. Since the MPs range extends to micrometer particle sizes that are challenging to sample and for which contaminant concentrations cannot easily be

measured today, the possibility to infer contaminant concentrations in the MP polymers from environmental concentrations may prove useful. In addition for chemicals that adsorb rather than absorb to the polymers, estimates should be based on surface area-based data rather than mass or volume-based amounts of plastic transported (Nerland et al., 2014). Nonetheless, this mode of transport maybe significant for substances that do not necessarily demonstrate possibility for long-range transport through other more common transport processes.

If a contaminant concentration gradient between the organism and the ingested plastic exist, gut fluids have the potential to facilitate the transport of chemicals from the plastic to the organism (Nerland et al., 2014). While laboratory studies have demonstrated that transfer of chemicals from ingested plastic particles to the organism is possible, whether the magnitude of this process is sufficient high to influence bioaccumulation and magnification of these chemicals into organisms and food chains is not certain (Browne et. al., 2013; Rochman et. al., 2013; Wright et. al., 2013). Contaminant concentrations on plastic debris and particles sampled from beaches may be representative of concentrations in their surroundings. Particles from heavily polluted areas contain higher concentration than those from less polluted areas (Free et al., 2014; Veerasingam et al., 2016). In the absence of information on mechanism of action these chemicals for ingested MPs towards human, it is impossible to quantify the level of pollutants under MPs.

## **CHAPTER 6**

### **CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH**

#### **6.1 Conclusion**

In conclusion, brand X and Y manufactured for poultry and aquaculture feedstock are contaminated with MPs and thus implies that marine environment where fishes were caught is polluted with MPs. This study data is also consistent with earlier studies that recorded prevalence of similar morphology and plastic polymers. There is also chance of MPs to enter human tissue via diet intake of edible tissue of contaminated poultry or marine biota. However, there is a limitation of study due to technical limitation which is the use of filter paper with pore size of 149  $\mu\text{m}$ .

## **6.2 Recommendations for Future Research**

### **1. Using smaller size of filter membrane**

Due to the usage of 149 $\mu$ m filter membrane for first filtration, they were chances of loss of microplastics and thus, reduced the number of isolated particles. Hence, for future research, it is recommended to use a smaller size of filter membrane.

### **2. Using fish muscle as the raw materials of fishmeals instead of fish waste**

Fishmeals that were made up of fish muscle can give indirect insight on the number of microplastics in fish tissue and enable the researchers to investigate the possibility of translocation of microplastics to the fish tissue.

### **3. Regularly monitor the level of microplastics in fishmeals**

As this study was only the first phase to investigate the presence of microplastics in fishmeals, hence it is recommended that further study is conducted to monitor the level of microplastics in fishmeals in order to create data for epidemiological studies.

#### **4. Regularly monitor the level of microplastics in farmed animals**

**As it was confirmed that there were presence of microplastics in fishmeals, it is recommended that the further study is conducted to monitor the level of microplastics in farmed animals.**



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