



Faculty of Resource Science and Technology

**Ingestion and Types of Microplastic in *Meretrix lyrata* from Buntal Bay
Sarawak through Nile Red Fluorescent Tagging**

Eric Lau Ngie Hao

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Ingestion and Types of Microplastics in *Meretrix lyrata* from Buntal Bay
Sarawak through Nile Red Fluorescent Tagging

Eric Lau Ngie Hao

A thesis submitted

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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Signature

Name: Eric Lau Ngie Hao

Matric No.: 18020217

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

Date :

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ABSTRACT

Microplastic had emerged as new form of plastic pollution. Many ingestions in biota were reported worldwide. Filter feeder such as bivalves are prone to microplastic ingestion. Shellfish were usually consumed as whole, without gut removal. This poses a threat towards human consumption. In this study, hard shell clam (*Meretrix lyrata*) was chosen and collected 4 times (2 times in both October 2020 (pre-monsoon) and March 2021 (post-monsoon) from local fish market (Kampung Buntal Wet Market) to investigate the factor of size class and harvesting season towards the ingestion rate of *Meretrix lyrata*. Fluorescent tagging using Nile red dye were used to aid the quantification process. Polymer identification were done on isolated suspected particles using Nicolet IN10 μ -FTIR and compared to an open-source FTIR reference library OpenSpecy. All samples (81 samples) in this study were positive with microplastic ingestion. A total of 15867 microplastic were quantified, the most dominant shape type is particle type (89%). The average microplastic size was 0.043 mm - 0.069 mm, and average of 195.90 ± 43.6 microplastic/individuals. Results shows that smaller sized clam tends to ingest more microplastic compare to larger sized clams. There were no significant differences ($p= 0.05$) in microplastic ingestion according to harvesting season. This might indicate that habitats for *M. lyrata* have pervasive microplastic pollution. PVC, PP with silicate mix, resin dispersion and PDMS were the four polymers identified from the microplastic particles isolated. All four were common polymers that can be found at place with human settlements. Fluorescent tagging using Nile red is a good method in quantification of microplastic however modification and precautionary steps were needed to obtain optimum results are important for microplastic detection improvements.

Keywords: *Meretrix lyrata*, nile red fluorescent tagging, microplastic. μ -FTIR, size-dependent

Dokumentasi Jenis dan Jumlah Mikroplastik dalam Meretrix lyrata dari Kampung Buntal, Kuching, Sarawak melalui Penandaan Pendarfluor menggunakan Nile red

ABSTRAK

Mikroplastik telah disahkan sebagai punca utama pencemaran plastik. Sehingga kini, terdapat banyak penerbitan mengenai perjumpaan mikroplastik dalam usus hidupan laut. Organisma penyuap penapis seperti kerang-kerangan merupakan hidupan laut yang kerap dilaporkan perjumpaan mikroplastik dalam usus. Kerang-kerangan seringkali dimakan secara menyeluruh tanpa dibuang sistem usus. Ini merbahaya kepada manusia sebagai kosumer kerang-kerangan kerana mikroplastik memasuki tubuh manusia melalui pemakanan. Dalam kajian ini, Kepah (*Meretrix lyrata*) dari Kampung Buntal dikaji untuk memastikan pengaruhan saiz kepah dan musim tengkujuh terhadap jumlah mikroplastik dalam usus kepah. Penandaan pendarfluor menggunakan Nile red adalah metodologi utama untuk kajian ini. Pengenalpastian polimer dijalankan dengan penggunaan mesin Nicolet IN10 μ -FTIR dan sumber rujukan terbuka OpenSpecy. Mikroplastik telah dijumpai dalam kesemua sampel kajian ini. Sebanyak 15867 butiran mikroplastik dijumpai dan plastic berbentuk partikal merupakan majoriti (89%). Saiz purata mikroplastik adalah antara 0.043 mm sehingga 0.069 mm, dan jumlah purata mikroplastik adalah 195.90 ± 43.6 mikroplastik/individu. Keputusan menunjukkan bahawa kepah yang bersaiz kecil mempunyai lebih banyak bilangan mikroplastik dalam usus berbanding kepah bersaiz besar. Musim tengkujuh tidak mempengaruhi jumlah bilangan mikroplastik dalam kepah. Hal ini mungkin disebabkan habitat kepah yang mempunyai pencemaran mikroplastik. Beberapa polimer yang dikenal pasti hadir adalah PVC, PDMS, resin, serta PP. Keempat-empat polimer adalah polimer yang kerap dijumpai di kawasan kediaman atau perpopulasi

manusia. Penandaan pendarfluor menggunakan Nile red adalah metodologi untuk kuantifikasi yang baik serta sesuai untuk kuantifikasi mikroplastik dalam kerang-kerangan. Pengubahsuaian metodologi seharusnya dilakukan untuk mendapat bacaan tepat serta optimum bagi sampel kerang-kerangan.

Kata kunci: Meretrix lyrata, nile red penandaan pendarfluor, mikroplastik, μ -FTIR, bergantung kepada saiz

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LIST OF ABBREVIATIONS

FTIR	Fourier-transform infrared spectroscopy
HDPE	High density polyethylene
LDPE	Low density polyethylene
PDMS	Polydimethylsiloxanes
PET	Polyethylene terephthalate
PP	Polypropylene
PVC	Polyvinyl chloride

CHAPTER 1

INTRODUCTION

1.1 Introduction

Plastics is currently the most used material worldwide. Plastics Europe (2021) reported global plastic production had increased since 2016 and reached 367 mt in 2020. Characteristics such as low cost, strength, lightweight, corrosion resistance, durability, and good thermal and electrical insulation qualities increase the demand for plastics-based product productions. Asia alone had contributed 52% of the total plastic production in 2020 (Plastics Europe, 2021). Different plastic-based material variants produced have a finite lifespan and are quickly discarded. Most of the waste will either be dumped into landfills or littered into the natural environment, apart from specific recyclable variants. High environmental persistence of plastic causes it to be the top contributor to marine debris (Auta et al., 2017). While authorities and policymakers started working to deal with the marine debris issue, another ‘side product’ from marine debris received attention: microplastics (NOAA, 2020).

Lebreton et al. (2018) reported plastics as dominant type of marine litter within Great Pacific Garbage Patch. While the amount reported only consist of those positively buoyant plastic materials but the total plastic pollution maybe under-estimated as different types of plastic have different densities in nature. Lower density plastics such as polypropylene, polyethylene and polystyrene have higher transportability than those denser plastic type such as polyvinylchloride (PVC) which settle down but lower transportability due to certain natural events such as runoff from heavy rains or waves. (Woodall et al., 2014; Mani et al., 2015).

Back in 1972, Carpenter and Smith had first reported the presence of small fragment plastics but the microplastic related research only receive attention when large amount of microplastics were reported by Thompson et al. (2004) in sediment and plankton samples. The term microplastics was widely defined as plastic particles with a diameter smaller than 5 mm (Arthur et al., 2009), origin of these micro-sized particles can be defined as primary microplastic and secondary microplastic. Primary microplastic were defined as the plastic particles designed and produced intentionally as micro size. On the other hand, secondary microplastic were product from defragmentation of bigger plastic pieces from marine debris through processes such as mechanical degradation and photo-degradation.

Microplastic pollution has been reported in many marine ecosystems worldwide due to massive input from land source, the persistent of microplastic in environment and are readily to be transported by ocean current. (Lavers & Bond, 2017; Brach et al., 2018; Reed et al., 2018). Lebreton et al. (2019) suggested that buoyant macroplastic accumulate in offshore surface water should be lesser than predicted as a series of repeated activity like stranding, settling and resurfacing in coastal environment tend to trap most of the marine litters. This shows coastal area and estuarine as the second largest sink of microplastic. Usman et al. (2020) stated contribution of plastic marine debris of a country is highly depending on its population size and the efficiency of waste management system.

Seafood provides proteins, vitamins and other essential minerals for human diet (Jayasekara et al., 2020), discovery of microplastic in seafood poses high risk and the side effect of these anthropogenic particles needs high attention. Clams are one of the most popular seafood among Malaysian. It is a perfect vitamin, mineral and lean protein source for consumers. The non-selective feeding strategy of clams increases the abundance of anthropogenic particles such as microplastics.

Small size nature of microplastic increased the tendency of contaminants accumulate from the surroundings due to the hydrophobic nature and the high surface ratio properties (Shahul Hamid et al., 2018; Deng et al., 2021). Small size of microplastics increases the bio-availability for marine organisms. Bivalves are particularly interested in assessment of microplastic ingestion due to their extensive filter-feeding activity, through exposing them directly with microplastic in water column and sediment (Li et al., 2015). Several reports had report direct effect of microplastics on bivalves such as filtration activity and feeding behaviour (Rist et al., 2016; Green et al., 2017). Previous reports from Kolandhasamy et al. (2018) and Li et al. (2019) highlight three pathways of microplastic accumulation in mussels including ingestion, adherence and fusion. The high surface area to volume ratio of microplastic makes them a prefect vector for persistent organic pollutants (POPs) and heavy metals (Zhang et al., 2020). Apart from physical damage caused by microplastic ingestion, bioaccumulation of harmful substances such as persistent organic pollutants and heavy metals along the food chain should also be given attention (Fu et al., 2020).

1.2 Problem statement

Meretrix lyarata or commonly known as ‘Kepah’ or lyrate asiatic hard clams were one of the local favourite shellfish species. The clams can be easily obtained by the locals throughout the year. (either through the imported frozen clams or buy directly with local fishermen at study site Kampung Buntal). The common shell size of the clam (from imported frozen clam) is less than 50 mm (shell length), this draws the attention of current study to investigate the difference in microplastic contamination between different size class. The availability of the clams throughout the year regardless of seasons also draw the interest to investigate the microplastic detection within the clams between different harvesting season.

1.3 Objective

Current study aims to utilize fluorescent tagging method to increase the sensitivity and reduce the bias from the existing quantification method for microplastic quantification in biota samples. This study also aims to investigate different possible factors which could affects the microplastic ingestion rate in white hard clam (*M. lyrata*). Therefore, the objectives of this study are:

- 1) To combine and adapt different microplastic quantification method and merging rapid screening method using fluorescent tagging.
- 2) To investigate factors size and seasonality to amount of microplastic ingestion

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy and morphology

Meretrix lyrata clam belongs to family Veneridae which were reported inhabiting in South and Southeast Asia regions (Liu et al., 2006) and also in several locations within Malaysian waters including Sarawak, Malaysia such as current project study site Kampung Buntal and Kabong (Betong District) (Hamli et al., 2016). The key characteristic of *Meretrix lyrata* with other *Meretrix* sp. were the undulate pattern on the shell surface (Appendix 5). The classification of clam according to Nguyen (1996) was shown in Figure 2.1.

Phylum: Mollusca

Class: Bivalvia

Order: Venerida

Family: Veneridae

Genus: *Meretrix*

Species: *Meretrix lyrata* (Sowerby, 1851)

Figure 2.1 Classification of clam taxonomy

As one of the commercially important bivalve species, *Meretrix* sp. productions are highly dependent on natural sources. (Duisan et al., 2022). Both Hamli et al. (2016) and Desrita et al. (2019) reported the increase demand of *Meretrix* sp. clam in Southeast Asia and Malaysia.

The *M. lyrata* is described by Helm et al. (2004) as a clam with two equal shells, in which calcium carbonate was the main component of the shells. The connecting side of the two shells which known as umbo were the dorsal part of the clam. Siphons were at the posterior side of the clam and foot were at the anterior side of the clam. Figure 2.2 shows the internal surface view and dorsal view of *M. lyrata*. Duisan et al. (2022) explained *M. meretrix* with size between 50mm-70mm is matured enough to be induced for spawning.

The development stages of *M. lyrata* includes embryonic stage, D-larve stage, mature larvae, early juvenile and spat. (Helm et al., 2004). Sandy tidal flats were the optimum growing environment for *Meretrix* sp. with the sediment composition of 80 - 90% sand and 9 – 14% mud and organic fragments. (Oanh & Lap, 2020). Sediment dwelling nature of the clam poses this species under threat with exposure towards vast amount of microplastics found within coastal sediments. (Urban-Malinga et al., 2021).

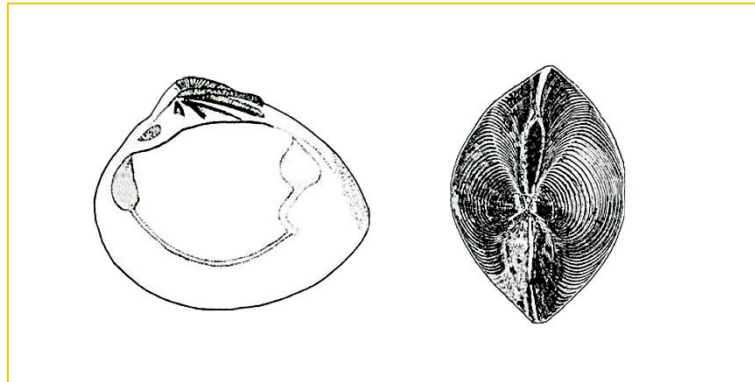


Figure 2.2 *M. lyrata* internal surface of right valve view (left) and dorsal view (right)

2.2 Plastic and its emergences

Plastic had become most of the substitute material due to its durability, versatility and cheap in production. (Klemeš et al., 2020) Packing industry is one of the industries that uses plastic material the most. Plastics are products of monomers connected repeatedly through polymerization and forming large molecular chain. The combination of different

monomers is possible and gives the end product both of the characteristics of the monomers. This widely increase the uses of plastics material in our daily life. Ranging from car parts, building materials, packing, and down to microbeads in cosmetic products.

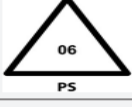
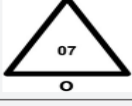
Plastic can be classified into two categories, thermosetting plastics and thermoplastics (Kazemi et al., 2021). Majority of thermosetting plastics such as bakelite are permanently mold, cannot be melted and reformed. This irreversible chemical change characteristic made thermosetting plastics non-recyclable. On the other hand, thermoplastics such as polyethylene terephthalate (PET) and polypropylene which are far more abundant are recyclable by heated until melted and remolded to form into new shapes.

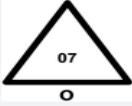
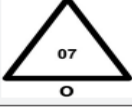
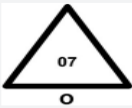
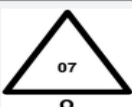
In 2021 ASTM International revised the symbols from the code with ASTM D7611 standard, replacing the familiar three chasing arrows with solid equilateral triangles (ASTM International, 2021). The updated version of the ASTM International codes was shown in Table 2.1. This is to prevent the confusion of public because the old symbol looks similar to universal recycling symbols.

Table 2.1 Type of plastic according to ASTM designator code and its application

American Society for Testing and Materials designator code	Plastic	Abbreviation	Typical application
	Polyethylene terephthalate	PET	Beverage bottles Food containers Film and sheeting Strapping Fibres Fleece Filling material
	High-density polyethylene	HDPE	Chemical containers Beverage bottles Food containers Crates Buckets Tubing Pipes
	Polyvinyl chloride (rigid)	PVC	Containers Electrical conduit Pipes Gutters Cladding Window frames
	Polyvinyl chloride (plasticised)	PVC	Cable insulation Garden hose Footwear Sheeting Flooring Mats
	Low-density polyethylene	LDPE	Bags Squeezable bottles Food packaging Carton linings Shrink wrap Pallet sheets Mulch film Waste bins Outdoor furniture

Table 2.1 continued

American Society for Testing and Materials designator code	Plastic	Abbreviation	Typical application
	Polypropylene	PP	Bottle caps and container lids Packaging tape Pipes Rope Automotive Applications Outdoor furniture
	Polystyrene (rigid)	PS	Disposable cutlery and tableware Rigid disposable food containers Other low-cost applications requiring rigidity
	Polystyrene foams (expanded or extruded)	EPS, XPS	Disposable food containers and cups Packaging foam Foam boxes Thermal insulation
	Polycarbonate	PC	Information storage discs Traffic light lenses Riot shields Security windows Eye protection
	Poly(methyl methacrylate)	PMA	Glazing Aircraft canopies and windows Fluorescent light diffusers Vehicle lighting covers and lenses Contact lenses

American Society for Testing and Materials designator code	Plastic	Abbreviation	Typical application
	Polytetrafluoroethylene	PTFE	Non-stick coatings for cookware and bakeware Breathable fabrics Plumbing tape Waterslides Low-friction coatings Automotive applications
	Acrylonitrile butadiene styrene	ABS	Electrical and electronic equipment Automotive applications Pipes
	Polyamide (nylon)	PA	Fibres Bristles Monofilament Bearings Automotive applications
	Polychloroprene (neoprene)	CR	Gloves Footwear Diving garments Medical bandages and supports Automotive gaskets Electrical insulation Weather balloons

2.3 Plastic after use and fate

Generally recycling of plastic is possible for certain polymer type which manufactured through injection molding at low temperatures, thermoplastics such as PET, PP, and HDPE. Recycling other waste material such as metal (80%), paper (60%), glass (50%) but recycling of plastic waste is only at 14-18%. (Haward, 2018; Hossain et al., 2021). One reason for the recycling industry does not receive much support and attention might be the confusion and misunderstanding of certain plastic are recyclable. (Worrell & Reuter, 2014). Acceptance of general public towards recycled plastic products is still low, reported by van der Vegt et al. (2022), general public's image towards both used plastics and perception of recycled material are associated with being dirty, unhygienic, unsafe or in lower quality. Another factor voiced out by van der Vegt et al. (2022) which hinder or disturb sorting of used plastic materials in recycling process is manufacture of the plastic products involves combinations of different materials, coloration, additives and adhesives. Xanthos (2012) reported addition of additive in polypropylene can increase the resistance of PP to oxidation.

A survey done by Dilkes-Hoffman et al. (2019) shows that most of the respondents are aware of high load of general waste will be landfilled and concerns about the amount of plastic waste that will be disposed in landfill. Taking the easiest example PET which is the plastic type for mineral water bottles, although recycling of PET is possible but people tend to dispose it as general waste. Food and drinking manufacturers refuse to use recycled products as there are possible contamination such as chemical additives, food residue and degraded product sorb on the plastic prior to recycling process.

The fate of these plastic waste will either end up in landfills or being burn to reduce the waste produced. (Boucher & Friot, 2017). However, burning of plastics could produce

harmful gases and chemical which give harmful impacts to human. Landfill was currently the most accepted way of managing plastic waste worldwide.

Kershaw and Rochman (2015) reported that land-based sources plastic waste was the main input of plastic waste into oceans over marine based sources. Evidence can be shown by the type of plastic during beach cleaning activities and other marine debris initiatives. (Agamuthu et al., 2019). Plastic pollution had been reported to cause harm to wildlife through entanglement and ingestion. It may also degrade coastal benthic habitats through processes such as smothering and changes the sediment permeability due to buried plastic. (Carson et al., 2011).

As reported by Jammbeck et al. (2015) and Lebreton et al. (2017) dispersion of plastic waste in open sea had hugely affected marine ecosystem. The durability and long-lasting characteristics of plastics-based products leads to huge amount of waste and mismanaging of these wastes will cause leakages to environment largest sink, the ocean. These plastic wastes will also undergo different weathering processes such as UV-photooxidation, mechanical abrasion, and hydrolysis breaking down into smaller pieces.

2.4 Microplastic and its abundance

The term microplastics was widely defined as plastic particles with a diameter smaller than 5 mm (Arthur et al., 2009), origin of these micro-sized particles can be defined as primary microplastic and secondary microplastic. Primary microplastic were defined as the plastic particles designed and produced intentionally as micro size. On the other hand, secondary microplastic were product from defragmentation of bigger plastic pieces from marine debris through processes such as mechanical degradation and photo-degradation.

These small pieces formed were termed as microplastics and even nanoplastics according to the size. The small size nature of these microplastics increased the

bioavailability in environment and are inadvertently consumed by marine organisms. The potential transference of microplastic through food chain had received high attention when organisms such as mussels and planktons were reported to ingest microplastics. (Cole et al., 2013). Farrell and Nelson (2013) proposed the possibility of indirect ingestion of microplastic through ingesting contaminated prey. Turner (2018) stated external condition such as pH, salinity UV light and shear stress can cause sorption and desorption of chemical on microplastic. Prunier et al. (2019) suggest harmful chemical uses microplastic as carrier and transported to marine environment.

Abundance of microplastics can be affect by the density nature of the plastic waste or the microplastic component. Overall pollution cannot be quantified but only be done through estimation through series of calculation and prediction. Leberton and the team did a series of predictions through existing data to create estimation model to understand river plastic inputs into ocean (Lebreton et al., 2017), accumulation of plastic in Great Pacific Garbage Patch (Lebreton et al., 2018) and mass budget for positively buoyant macroplastic debris in the ocean (Lebreton et al., 2019). Most of the river input are from Asia, this might due to the rapid development and economic growth but poor waste management. Jambeck et al. (2015) reported similar trend, 6 out of the top 10 countries based on the mass input of mismanaged plastic waste from coastal population are from Asia. Lebraton et al. (2018) reported the evidence of plastic waste invading in the Great Pacific Garbage Patch, plastic was the dominant type of marine litter within the patch. It was reported 79 K tonnes of plastic mass within GPGP and 22 % from the total mass was microplastic and mesoplastic. Lebraton et al. (2019) hypothesized the missing amount of buoyant plastic should be settling at coastal environment through process such as stranding, settling and resurfacing process. All these evidence shows the availability of plastic in different form are invading our environment.

These data were not inclusive to those which had been ingested or those which sinks to deeper part of the ocean due to density

2.5 Threat of microplastic

Microplastic are suggested to be vector of toxic substance due to the hydrophobic properties. Contaminants such as Bisphenol-A, alkylphenols, PAE, phthalates, organobromine, styrene and heavy metal could sorb on the surface of microplastic and desorb the contaminants when entered into biotic system (Wright et al., 2013; Yi & Kannan, 2016). Many laboratory experiments were done to test the adverse effect of microplastics towards test subject such as fish and bivalves. Urban-Malinga et al. (2021) reported change of behavior in two sediment-dwelling bivalves, cockle (*Cerastoderma glaucum*) and Baltic clam (*Limecola balthica*) after exposed to PE microspheres. *C. glaucum* were reported with lesser emergence and in lower number and *L. balthica* shows avoidance behavior by buried deeper into the sediment after exposure of PE microspheres.

Different exposure experiments were designed to test the influence from contaminants such as dichlorodiphenyltrichloroethanes (DDTs), polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) towards the ingested samples such as fish and bivalves. Karabalaie et al. (2021) reported addition of polystyrene microplastic treatment and chlorpyrifos (CPF) alone alters histological and histomorphometrical parameters in gill, gut and skin of juvenile *Onocorynchus mykiss* (rainbow trout) after 96 h exposure. The alteration is worse when the mixture of MP-PS and CPF was introduced to the test subject (*O. mykiss*).

Lu et al. (2016) reported observation such as inflammation, hepatic steatosis in liver, oxidative stress and altered metabolomics profiles in Zebrafish (*Danio rerio*) after exposure of polystyrene microspheres for three weeks.

Cho et al. (2019) reported a 95% detection of microplastic contamination at a market-based survey for 4 popular bivalve species (clam, mussel, oyster and scallop) in South Korea. These bivalves are usually eaten whole or without gut removal, this can be a possible route of human exposure to microplastic.

2.6 Selected novel method and problem faced

Pretreatments were usually done towards environmental samples for separation of impurities especially for biota samples in which the common impurities are biological material, biofilms, algae and detritus (Cole et al., 2014). Common pretreatment methods used for biomaterial removal (purification of samples from organic matter) are acid digestion, alkaline digestion, oxidizers or enzyme digestion. (Enders et al., 2016; Zobkov & Esiukova, 2018). Hydrogen peroxide with addition of Fe (II), Fenton's reagent was used to increase the removal rate of organic matter from organic rich sediments (Vermeiren et al., 2020). Oxidative approach using hydrogen peroxide is recommended only when dealing with samples that are highly contaminated with organic material. Nuelle et al. (2014) reported the reduction in recovery of nylon and also reported change of color while treating PET samples with an oxidative approach using hydrogen peroxide (H₂O₂).

Cole et al. (2014) reported the optimized enzymatic digestion method (proteinase K) has a higher recovery rate compared to acidic (HCl) and alkaline digestion (NaOH) approaches. Karlsson et al. (2017) adapted the enzymatic digestion method (proteinase K) with CaCl₂ as catalyst and added an oxidative method (30% H₂O₂, overnight) to remove excess chitin. This method gives a high recovery rate (97%) but the drawback is the cost of the method is expensive.

Karami et al. (2017) tested different existing organic matter purification protocol (acid digestion (HCl, HNO₃), alkaline digestion (KOH, NaOH, NaClO) and oxidative digestion (H₂O₂)) on fish samples and concluded that alkaline digestion using 10% KOH under 40°C provides the optimum result from the aspects of organic matter removal and morphological changes on targeted microplastic samples. Therefore, alkaline digestion using 10% KOH under 40°C is selected for the current project.

One of the crucial steps in microplastic detection is isolating microplastic particles from a complex matrix (environmental samples, biota samples). Density separation is a method which benefits from the different density nature of plastic material. (Zarfl, 2019). The density medium used will have an important role in ensuring the success separation of microplastic particles for the tested environmental matrix. Quinn et al. (2017) evaluated several density separation medium ranging from tap water to different salt solution (tap water 1.00 g cm⁻³, NaCl 1.17 g cm⁻³, NaBr 1.37 g cm⁻³, NaI 1.57 g cm⁻³, ZnBr₂ 1.71 g cm⁻³) and reported ZnBr₂ having the higher recovery rate among the tested separation medium. The density of common plastic ranges between 0.9-1.5 g cm⁻³ (Quinn et al., 2017) therefore sodium iodide (NaI) and zinc bromide (ZnBr₂) are dense enough as the density medium for microplastic density separation. Zinc chloride (ZnCl₂) was also preferred as an alternative due to the density (~1.40 g cm⁻³) and reusing of chemicals through careful filtration and clean-up. (Maes et al., 2017). ZnCl₂ is selected as the density separation medium for the current project due to the capability of reuse.

After purification or pretreatment of the samples, the supernatant or solution will be isolated through filtration. The filter used should be made of non plastic materials such as glass fiber or aluminum oxide filter.

The two selected protocols (alkaline digestion using 10% KOH at 40°C and density separation using ZnCl_2 $\sim 1.40 \text{ g cm}^{-3}$) were performed as the pretreatment towards biota sample before microplastic isolation through filtration using glass fiber filter paper.

Direct combination of the two protocols was not possible without slight modification as precipitation is formed when both solutions are mixed creating a white precipitate zinc hydroxide (ZnOH_2). The adaptation method to overcome the mentioned issue is discussed in this thesis.

After purification, the samples isolated are ready to be quantified and further isolated per piece for qualification analysis. Optical techniques such as visual identification with either naked eye or stereo microscope were the most common methods applied in general. (Zarfl, 2019). Quantification of microplastics using visual identification requires highly experienced operators to justify the nature of the isolated particles and quantify the total isolated microplastics. Visual identification still has the risk of both misidentification and underestimation of microplastic as size limitation due to the resolution from microscope and not all plastic types have definite characteristics which can be observed visually. Löder & Gerdtz (2015) reported after reanalyzing pre-identified plastic particles through visual identification using a focal plane array detector-based micro fourier transform infrared (FTIR) spectroscopy showing only 1.4% of the particles identified were made from plastic material.

There are more sophisticated methods that can help to determine the nature of the isolated particles such as using Fourier transform infrared (FTIR) spectroscopy and Raman micro-spectroscopy.

FTIR operates by collecting the spectral of the test samples depending on three different modes namely reflectance, transmittance and Attenuated Total Reflectance (ATR). Reflectance mode can be used to collect spectra of irregular shaped microplastic (Levin & Bhargava, 2005). Transmittance mode collects total absorption of the test materials but is limited to certain thickness of microplastic samples. (Löder & Gerdts, 2015). Micro-ATR with combination of microscopy enables direct measurement as IR spectra were collected on the surface of the particle (Wagner et al., 2017).

On the other hand, Raman micro-spectroscopy is utilizing the measurements of wavelength and intensity of inelastically scattered light from molecules sourcing from a monochromatic light. Raman measurements allow identification of thicker and stronger absorbing particles compared to FTIR as Raman measurement is not dependent on the transmission of exciting light through the sample material (Lenz et al., 2015).

2.7 Application of rapid screening method using Nile red

The idea of using staining dye to aid in microplastic quantification was first suggested by Andrady et al. (2011). Maes et al. (2017) and Prata et al. (2019) tested different dye namely Oil red EGN, Eosin B, Rose Bengal, Hostasol Yellow 3G and Nile red. Prata et al. (2019) added an extra manipulating factor for testing (excitation wavelength for Nile red dye) and concluded 254 nm and 470 nm as the best excitation wavelength to identify synthetic materials.

Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) is a lipophilic fluorescent dye which has high chromophore sensitivity to solvent polarity which gives high quantum yield in non-polar media. (Martinez & Henary, 2016). Nile red is highly soluble and shows strong fluorescence in a wide range of organic solvent (Sitepu et al., 2012).

Introducing the Nile red dye staining step helps in increasing the precision of the microplastic quantification and identification through visual identification. Purification of the samples is very crucial if fluorescent tagging steps were adapted into normal protocol. Maes et al. (2017) reported observations as crab claw fragments show dull orange fluorescence under 470 nm ~ 540 nm excitation wavelength. Tamminga et al. (2017) also reported similar observations while comparing three different solvents (acetone, chloroform, n-Hexane) for extraction of Nile red dye under UV light source (365 nm). Different types of solvents for Nile red cannot overcome the issue of biogenic matter staining, so proper removal of organic matter is important prior to incorporating fluorescent tagging methods into existing protocols.

Prata et al. (2019) suggested 1 $\mu\text{g ml}^{-1}$ of working concentration (prepared in ethanol), 30 min of exposure period and excitation wavelength of 470 nm with orange filter equipped. Maes et al. (2017) reported 10 min exposure period can also provide similar result, therefore the selected conditions for Nile red staining for current project are 1 $\mu\text{g ml}^{-1}$ of working concentration (prepared in ethanol), 10-30 min of exposure period and excitation wavelength of 470 nm with orange filter equipped. A summarized novel method of rapid screening method using Nile red is shown in Figure 2.3.

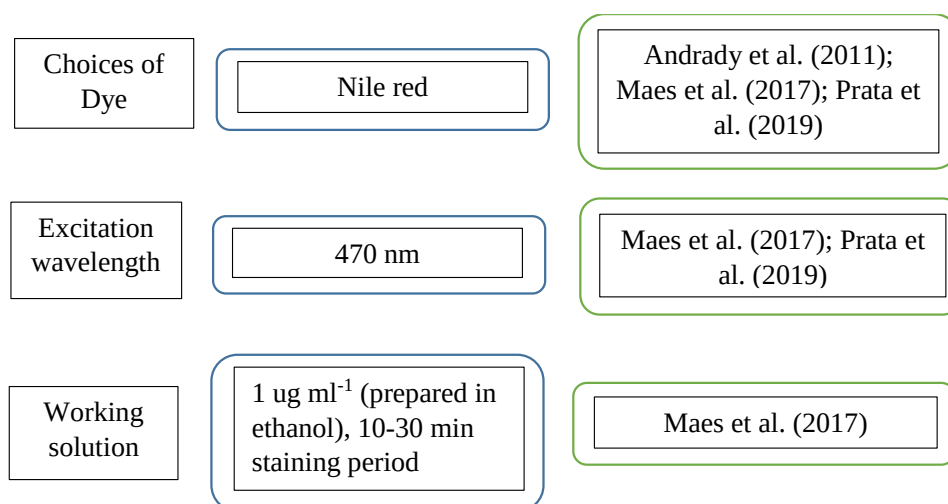


Figure 2.3 Summarize novel method in application of Nile red in rapid screening method.

2.8 Reducing microplastic contamination prior to consumption

Bivalves collected from the wild and cultured were reported contamination of microplastic. Researchers suggested the depuration method can reduce the microplastic concentrations in bivalves. Baechler et al. (2019) suggested depurating both pacific oyster (*Crassostrea gigas*) and razor clam (*Siliqua patula*) in freshwater for a short period of time can reduce anthropogenic debris in the seafood. Cauwenberghe & Janssen (2014) reported depuration of *Mytilus edulis* and *Crassostrea gigas* with artificial seawater for 3 days successfully removed the larger ingested microplastics Wang et al. (2021) did a similar depuration experiment on *Mytilus coruscus* (maximum 87 hours), and reported similar excretion trend as reported by Cauwenberghe & Janssen (2014), the larger microplastics were excreted during depuration and further observed translocation of microplastic particles in vivo as detection of microplastics at mantle of mussels were detected late after exposure and depuration. Hamid et al. (2020) reported a decrease of microplastic (34%) in *Meretrix lyrata* after 3 days depuration period. The team also reported the uptake rate of *M. lyrata* will reduce when the maximum capacity of uptake is reached. Therefore it is recommended

that given depuration towards live bivalve prior to be processed for consumption does help to reduce the microplastic content within the bivalve.

CHAPTER 3

METHODOLOGY

3.1 Study site

The study site is located approximately 25 km north of Kuching. It has a coastline of 7.5 km which ranges from Tg. Batu headland in the northwest to Muara Buntal in the southeast. Figure 3.1 shows the location of the study site. The wide intertidal zone within the coastline is one of the unique geomorphology of the coastline, the delta region also provides perfect habitat for different organisms such as Irrawaddy dolphin, razor clams, lyrate asiatic hard clam and mud crabs.



Figure 3.1 Map of study site Kampung Buntal, Sarawak with land use along the river to the delta region of Buntal river.

The delta is connected by the Buntal river, with mangrove forest along the river but the delta region is constantly experiencing sedimentation. This provides mudflat and widens the intertidal zone, providing habitat for migratory birds and bivalves. High tidal activity

causes the erosion therefore artificial coastal protection was built to protect the village from further erosion. Local villagers will collect bivalves such as lyrate asiatic hard clam and razor clam. Hamli et al. (2016) reported Buntal bay as one of the famous habitats for *M. lyrata* in Sarawak region.

3.2 Sample collection and processing

Lyrate asiatic hard clams (*M. lyrata*) were bought during visit attempts to the sampling site (Kampung Buntal). Two attempts per season (October 2020 and March 2021) (pre-monsoon season and post-monsoon season) were done throughout the project study period. The seasons (pre-monsoon and post-monsoon) were determined according to the dates provided by Metrology Malaysia (Appendix 2). The sizes and the amount of clam were picked randomly to ensure sample randomness. Samples were sent back to the lab and kept in -20 °C freezer before carry forward for further analysis.

One-way airflow condition in the lab was created through adjusting experimenting location under an air-conditioner flow. The lab was pre cleaned and surfaces were wiped with 70% ethanol using a cotton cloth. A full cotton lab coat and nitrile gloves were worn while handling the experiment. All solutions prepared and used (distilled water, 10 % KOH, ZnCl₂, 10 % HCl) were pre filtered with 0.5 µm Whatman GC50 filter paper to prevent contamination of microplastic from other sources. All filter papers used in this project were GF/C filter paper (ADVANTEC GC-50, 0.5 µm). The apparatus material used was either glass or stainless steel. The glassware and stainless steel tweezer were pre cleaned filtered deionised water and rinse with filtered 70% ethanol.

The clams were thawed at room temperature prior to cleaning the shell surface to remove the possible contaminations from the outer shell using filtered distilled water. The

flesh of clam was removed using a stainless steel tweezer after obtaining the morphometric data (shell length and shell height) using an electronic caliper ($\pm 0.01\text{mm}$). The flesh were then kept in a glass jar for further pre-treatment of the biota samples prior to microplastic quantification.

3.3 Methodology adaptation

Direct application of compiled novel methods didn't produce satisfactory results as expected (Refer Appendix 3 for insights of results obtained from compiled novel method). 66% from the total samples collected were used for methodology adaptation from compiled novel methods. Methodology discussed beyond this section will be method after adaptation.

3.3.1 Digestion method

The role of digestion protocol is to remove biological substances leaving behind non-biological substances to ease in either quantification or qualitative analysis of microplastic. 10 % Potassium hydroxide (KOH) was prepared through dissolving 100 g of KOH pellet in 1000 ml of distilled water. 100 ml of 10 % KOH were poured in with the dissected clams and incubated under 40 °C in the Microbial incubator (Astral, OBI STD 42). The end point of the digestion was set as no clam tissue was observed in the mixture. The incubation period was extended (ranging from 24 ~ 72 hours) until the end point of digestion was reached.

3.3.2 Density separation

The key role of applying density separation in microplastic quantification is to reduce the possible interference from other non-plastic substances such as shell, wood, sand, clay, etc. Density separation allows all the substance or particle less dense than the density medium to float. The role of density separation is to purify environmental samples through floatation and isolate the desired items according to the density difference between the

density medium used and the density of the isolated item. Zinc chloride (ZnCl_2) was selected as the density separation medium for the current project due to cheaper cost and the ability of ZnCl_2 in recycling of used chemical through filtration and maintaining the density through addition of pure ZnCl_2 . ZnCl_2 stock solutions were prepared with 1:1 ratio of ZnCl_2 pellet and distilled water. Density was checked before filtration and after to ensure the working solution density was between 1.40 g cm^{-3} (± 0.02) through measuring a 10 ml of prepared solution using an analytical balance.

The digested matrix from the digestion protocol was mixed with ZnCl_2 solution after acidification with 10 % HCl. The mixture was then transferred to a self-made density separator unit, (designed origin from Maes et al., 2017). The mixture was mixed evenly through manual shaking and covered with small pieces of aluminum foil before being left for 24 hours for the substance within the mixture to separate. The valve was closed before removing the upper solution together with the floating microplastics and filtering to a clean filter paper. 10 % HCl was used to rinse the top part of the density separator unit to ensure all floating substances were filtered to the filter paper.

All rinsing solution was replaced with 10 % HCl to avoid precipitation. The used ZnCl_2 (ZnCl_2 from the lower part of the density separator) were then collected and filtered using GF/C filter paper (ADVANTEC GC-50, $0.5 \mu\text{m}$). The collected ZnCl_2 were measured density and the density is adjusted through adding of pure ZnCl_2 .

3.3.3 Fluorescent tagging using Nile red

Nile red stock solutions were prepared in propanol with the ratio of 1:100. 0.06 grams of Nile red powder were measured and mixed with 60 ml of propanol. The stock solution was then stored in a 60ml Scott bottle pre-wrapped with aluminum foil and kept at 4°C . The

stock solution was diluted 100 times in filtered distilled water to make a working solution. (1 mg mL^{-1}). After filtering the ZnCl_2 containing separated microplastic, the filtrate was pre-washed with a small amount of 10 % HCl before adding 10 ml of Nile red working solution. The Nile red was rested for 10-15 min for optimum staining result. After the staining period, the excess solution was filtered and washed with a small amount of filtered distilled water to wash off excess Nile red working solution.

3.3.4 Image processing

The stained samples on filter paper were transferred to the automated filter-scanning rig designed by Maes et al. (2017). (Appendix 1). It is a combination of a commercial micro-milling machine with a DSLR camera. The camera was controlled via remote shooting software, to optimize photo condition, the ISO, F-stop and exposure time were tuned to archive a dim background to present optimum result because most of the microplastic were smaller than the testing particles and not visible under bright background conditions. G-code routines provided were used to control the scanning. The 47 mm filter paper was separated into 24 parts and capturing of each part were controlled by the G-code. This was to provide a detailed image with minimum noise to reduce the counting error for the quantification step. The samples were viewed under blue light (Crime Lite: 450-510 nm) through an orange filter (529 nm). Before starting the scanning routine, the image quality was set to achieve a dim background with fluorescent particles retaining their fluorescence through tuning three different settings namely ISO, F-stop and exposure time. This was to provide a detailed image with minimum noise to reduce the counting error for the quantification step. The photos were then processed using Image J. Merging 24 photos using montage and the outcome was saved in .tif format.

3.3.5 Automated counting using MP-VAT

Manual counting after applying fluorescent tagging is archivable but there is another alternative which is an automated counting script developed by Prata et al. (2019). Three macros based on Image J application were developed by Prata et al. (2019), namely MP-VAT, MP-SCALE and MP-ACT. These three different macros have different functions. MP-VAT is the main macro plugin used for automated quantification of processed images. This automated software as claimed by Prata et al. (2019) had a recovery rate of at least 89 % which gives this software a reliable method for quantification.

MP-SCALE as the macros plugin which sets the scale for particles, which will later calibrate the results value generated to the unit of millimeter (mm). MP-ACT is a plugin which allows users to set different settings for quantification of microplastics. For the current project only MP-VAT and MP-SCALE were used.

MP-SCALE were used to set scale based on the known distance (diameter of the filter paper, 47 mm) before continuing with MP-VAT. This sets the scale from pixels to approximately 51 px mm^{-1} , this is to set the lower limit of the particles to less than 0.05 mm. This reduces the interference from small fluorescents which can be residue of Nile red dye. The results automated from MP-VAT were saved in .xls file which contains the area, category data, ferret and min ferret data. All quantitative data were tallied using Microsoft Excel and carry on with statistical analysis.

3.3.6 Polymer identification

Samples were isolated from the filter paper using a larvae forceps with stereomicroscope using blue light as light source for visual aid in microplastic sample isolation. 20 isolated samples were placed on Si filter, and viewed under Nicolet IN10 IR

microscope. Si filter were chosen over silver membrane due to the higher filtration rate and clearer background difference to ease in locating of microplastic samples under Nicolet IN10. Resulting spectra was obtained under ATR mode with germanium (Ge) ATR tip equipped. Spectral range were set at 4000 cm^{-1} – 675 cm^{-1} , with resolution of 4 cm^{-1} and 32 scans s^{-1} . The obtained spectra were processed by OMNIC Picta software and primary polymer identification was done with OMNIC polymer reference spectra library. This additional step was to ensure the spectrum collected was the spectrum of the microplastic samples but not the Si filter. Irregular surface of the suspected microplastic particles can be relocated while ATR tip is lowered to have contact with the suspected particle and lead to wrong spectrum (background filter spectrum) were collected. Collected spectrums were collected in unit of transmittance (%) and saved as .csv file. The results and spectral data were continued with cross validating with an open-source database OpenSpecy. (www.openspecy.org; Cowger et al., 2021). A comparison between the polymer detected using different reference library were done to understand which database is more reliable to test for environmental data.

3.3.7 Statistical analysis

Morphometric data were recorded and rearranged using Microsoft Excel. Quantitative data from MP-VAT were also compiled and tabulated using Microsoft Excel to compare with different size class and different harvesting seasons. Normality tests were done prior to any other statistical analysis to determine data sets were close to normal or skewed data. Student's T test will be used as the parametric test if the data sets were normally distributed, Mann-Whitney U test will be used as the non-parametric test if the data sets were not normally distributed. Statistical test was conducted to analyze the significant differences

in quantity of microplastic ingestion by difference class size of bivalve and different season of bivalve harvesting.

CHAPTER 4

RESULT

4.1 Procedure testing and adaptation

A series of testing and method validation were done to ensure the methodology design can produce optimum results without bias.

4.1.1 Filter paper selection

Two different types of filter paper recommended from literature namely polycarbonate filter paper and glass fiber filter paper were tested. The result from the trial shows fluorescent staining does not show noise from other non-targeted material (non-plastic items). Figure 4.1 shows the fluorescent tagging testing result using polycarbonate filter paper and glass fiber filter paper.

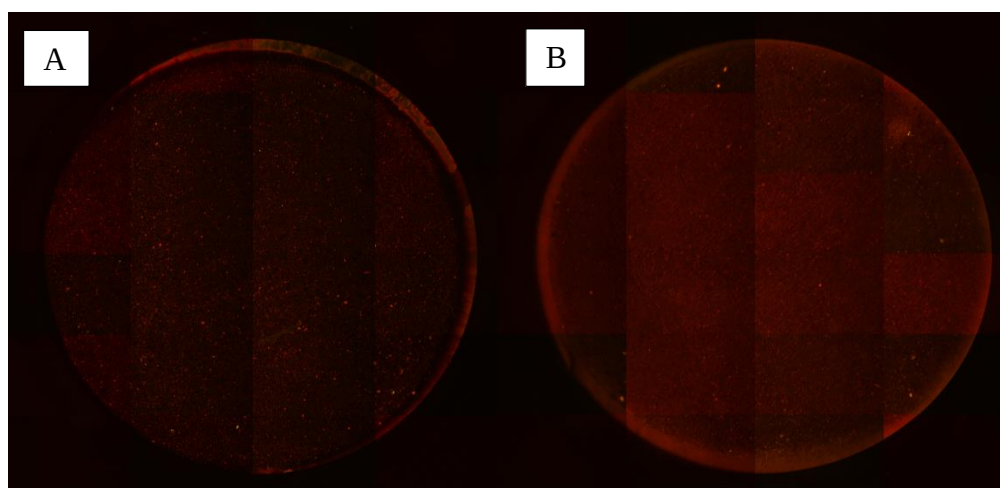


Figure 4.1 Fluorescent tagging testing using different type of filter paper, A: polycarbonate filter paper and B: glass fiber filter paper.

Stronger contrast between the fluorescent tagged particles and the background of the filter paper were observed in the polycarbonate filter compared to results shown using glass fiber filter. Minimal noise observed on glass fiber filter paper can be further minimized

through tuning the brightness and the contrast of the picture prior to automated quantification protocol. Furthermore, glass fiber filter paper is a cheaper and more readily available option compared to polycarbonate filter paper therefore glass fiber filter paper is selected as the main filter paper type for the current project.

4.1.2 Ability of Nile red in fluorescent tagging

Different types of plastic polymers (PET, PP, HDPE and PVC) were chosen to test the ability of Nile red fluorescent tagging. The plastic polymers were prepared in micro size and stained with Nile red at the same concentration as the current project (1 mgmL^{-1}). All 4 types of polymers show positive fluorescence after fluorescent tagging using Nile red protocol.

Different color and brightness of fluorescence were observed after exposure under blue light source (470 nm- 540 nm). Figure 4.2 shows fluorescent results of selected polymers after fluorescent tagging using Nile red. PET pellet, PP plastic rope and HDPE zip-lock bag shows a yellow fluorescent (under blue light emission and an orange filter) but at different brightness. PVC fragment shows a different color fluorescent (orange) compared to another three tested polymers.

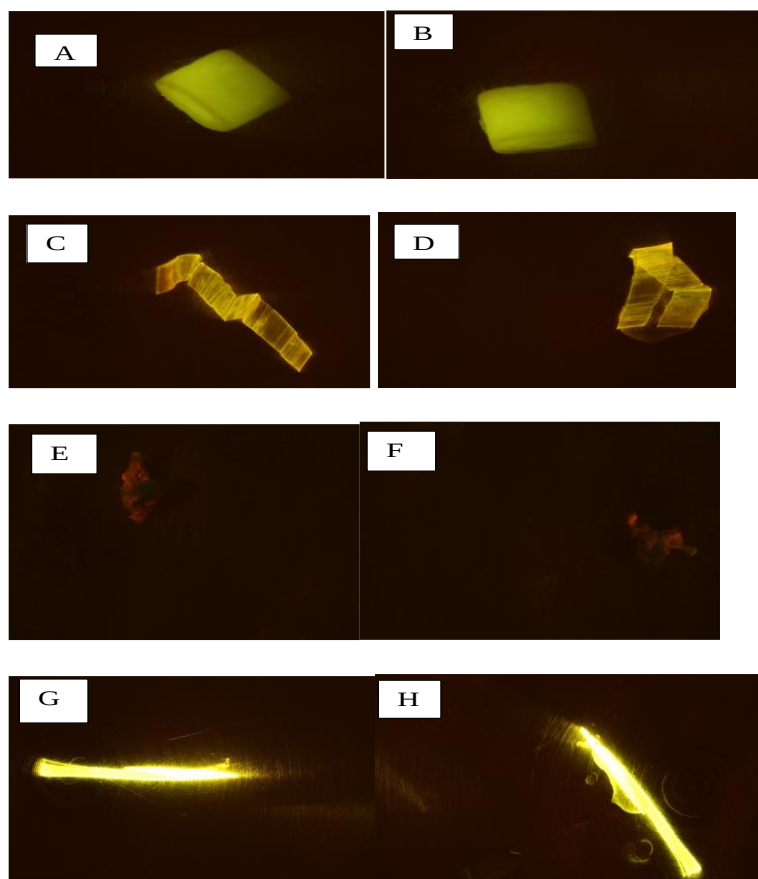


Figure 4.2 Plastic samples after staining with Nile red and viewed under blue light source with orange filter equipped. A: stained PET pellet before rinsing with filtered distilled water, B: washed stained PET pellet with filtered distilled water, C: stained PP plastic rope, D: washed stained PP plastic rope with filtered distilled water, E: stained PVC fragments, F: washed stained PVC fragments with filtered distilled water, G: stained HDPE ziplock bag pieces, H: washed stained HDPE ziplock bag pieces.

An additional rinsing step was proposed and tested. The aim of the rinsing step is to remove excess Nile red dye on the filter paper. The stained plastic particles were rinsed with filtered distilled water (non-organic solvent only) and viewed under the same condition to test the degree of fluorescence maintained or reduced. The result in Figure 4.2 shows the fluorescence effect before and after the rinsing step. There is only a slight reduction in the fluorescence intensity therefore the rinsing step is added into the methodology.

4.1.3 Method adaptation

The selected protocols for pretreatment of clam samples were alkaline digestion (10% KOH at 40°C) and density separation (ZnCl_2 1.40 g cm⁻³ (± 0.02)). Direct combination according to the sequence of digestion protocol followed by density separation. Direct mixture of solution containing microplastics after KOH digestion to ZnCl_2 causes precipitation and affects the density separation process. Figure 4.3 shows an example of precipitation formation after direct mixture of the two different solutions.



Figure 4.3 Snapshot of precipitation formed from direct mixture of digested solution (KOH) and density separation medium (ZnCl_2).

An additional step before mixing the two solutions is acidifying the digested solution after the end point is reached. The aim is to increase the acidity of the solution so no precipitation will form after mixing with zinc chloride solution. The acidify solution chosen is 10% HCl with the mixing ratio to digested KOH solution is 1.5:1. Lowering pH of the digested matrix prior to mixing with zinc chloride is important as the existence of water

could also cause precipitation (zinc hydroxide, ZnOH_2). Figure 4.4 shows staining results of samples isolated after density separation which are contaminated with ZnOH_2 .

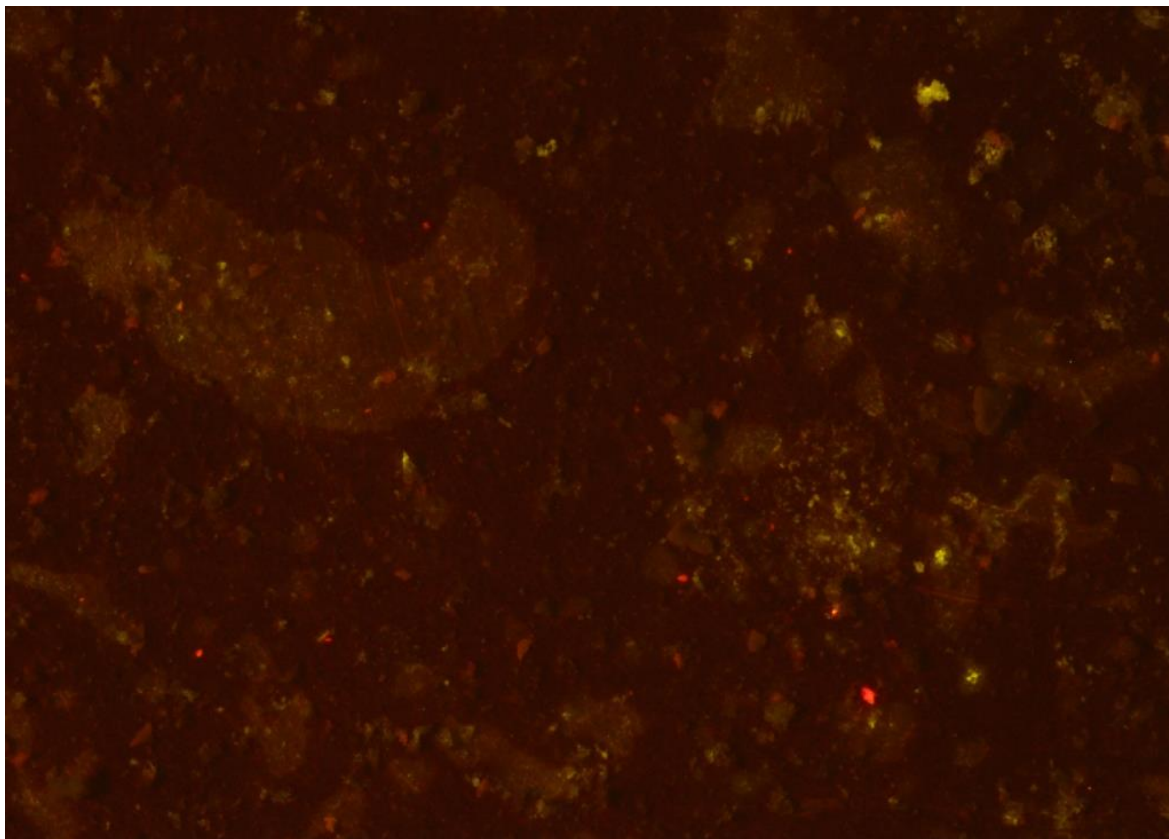


Figure 4.4 Staining efficiency of Nile red reduced due to presence of ZnOH_2 ‘masked’ the particles from being stained.

Existence of the precipitation can increase the difficulties in removal of excess Nile red dye after conducting fluorescent tagging protocol. As a precaution step to remove the small precipitation residue collected together after isolating the floating particles on the density separation medium, rinsing the filter paper with 10% HCl is performed before and after fluorescent tagging protocol, both followed with rinsing with filtered distilled water to remove the excess HCl and Nile red dye. Figure 4.5 shows the flow of methodology after adaptations were applied.

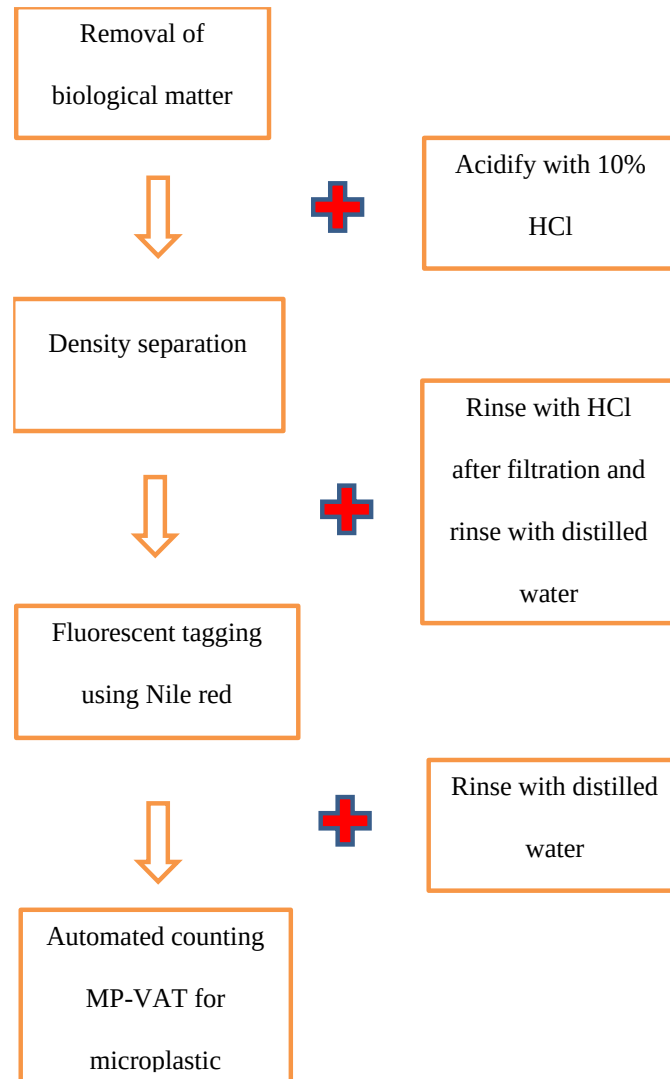


Figure 4.5 Methodology used after included adaptations to improve the sensitivity of microplastic detection and quantification.

4.2 Microplastic ingestion in *M. lyrata*

A total of 81 clams were investigated for quantification of microplastics. Figure 4.6 shows the scientific photo of *M. lyrata* and the identification of species were according to description explains by Hamli et al. (2016).



Figure 4.6 The scientific photo of *M. lyrata* collected from study site

The largest clam recorded was 76.14 mm (disc length), 63.56 mm (disc height) and the smallest clam recorded was 26.43 mm (disc length), 22.80 mm (disc height). All the sampled clams were positive with microplastic ingestion in which the quantity range of microplastic count was between 12~1047 microplastic/individual. The total microplastic detected were 15867 microplastics.

Automated script used (MP-VAT) categorized detected microplastic shape into three different categories (fiber, fragment, particle). The dimension of the microplastic is also recorded using MP-VAT script. Most of the microplastic detected were particle shape (14145 microplastics) followed by fragment (1518 microplastics) and fiber (204

microplastics). The dimension of the detected microplastic ranges from 0.008 mm (4 particles and 1 fragment) to 2.82 mm (fiber). All detected items were within the general category of microplastic (plastics with diameter less than 5 mm). Figure 4.7 shows the distribution of microplastic sorted according to shape from MP-VAT.

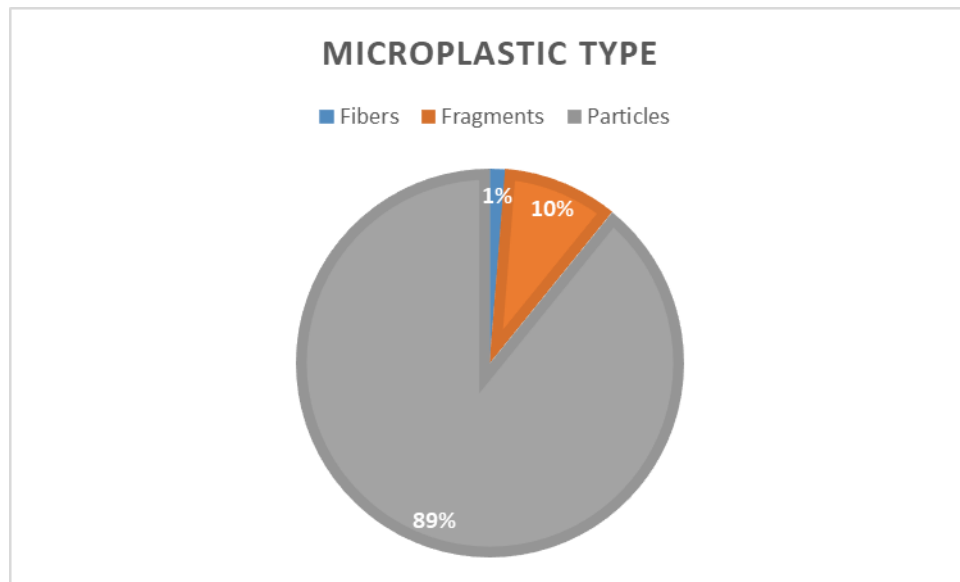


Figure 4.7 Distribution of microplastic sorted according to shape from MP-VAT

4.2.1 Microplastic ingestion according to factor: size class

The samples were rearranged according to each representative size class (20.0-29.99, 30.0-39.99, 40.0-49.99, 50.0-59.99, 60.0-69.99, 70.0-79.99, 80.0-89.99). Figure 4.8 shows the frequency of count according to *M. lyrata* clam size class (disc length).

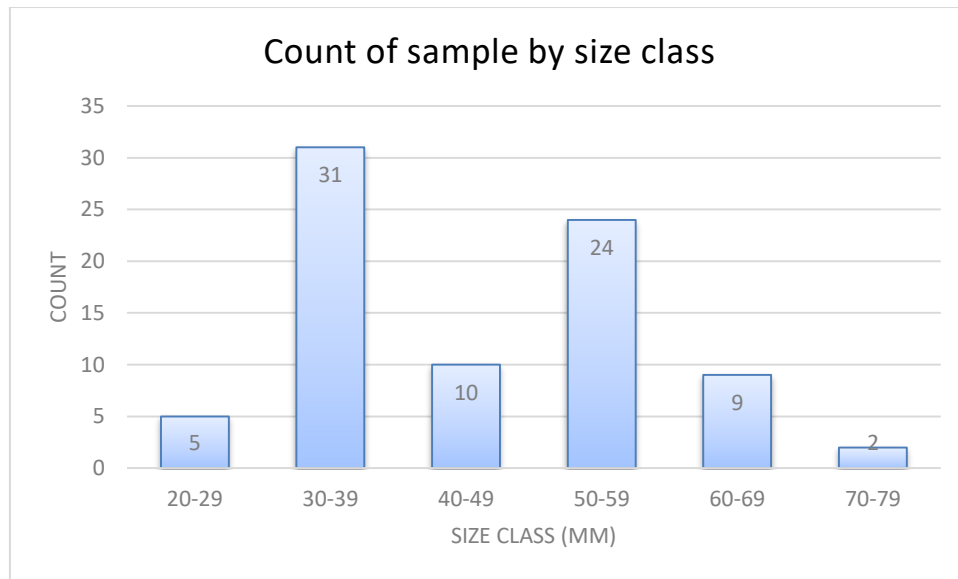


Figure 4.8 Frequency of count sorted according to size class of *M. lyrata*.

The microplastic ingestion in clams were further grouped into two different groups (0-49.99 mm) and (50.0-99.99 mm). After rearranging, there are a total of 44 samples in size class (0-49.99 mm) and 36 samples in size class of (50.0-99.99 mm). The descriptive analysis result between size class of (0-49.99 mm) and (50.0-99.99 mm) to microplastic ingestion were shown in Table 4.1.

Table 4.1 Descriptive analysis result for size class (0-49.9 mm) and (50.0-99.9 mm) to microplastic ingestion. (Retrieved from SPSS 28.0)

Descriptive				
Size (mm)				
Microplastic ingested	0-49.9	Sample size	44	
		Mean	218.6304	27.39305
		95% Confidence interval for mean	(Lower Bound) = 163.4580 (Upper Bound) = 273.8029	
		5% Trimmed Mean	196.2005	
		Median	150.5000	
		Variance	34517.438	
		Standard deviation	185.78869	
		Skewness	2.035	0.350
		Kurtosis	5.378	0.688
	50-99.9	Sample size	36	
		Mean	166.0000	34.43030
		95% Confidence interval for mean	Lower Bound = 96.0292 Upper Bound = 235.9708	
		5% Trimmed Mean	135.8968	
		Median	87.0000	
		Variance	41490.588	
		Standard deviation	203.69239	
		Skewness	2.805	0.398
		Kurtosis	9.956	0.778

The mean value for size class of (0-49.9 mm) is 218.63 ± 27.39 microplastic/individual. The highest microplastic count in the size class (0-49.9 mm) was 964 microplastic/individual (DL 38.77 mm, DH 33.43 mm) and the minimum count was 33 microplastic/individual (DL 45.83 mm, DH 40.06 mm).

The mean value for size class of (50.0-99.9 mm) is 166.00 ± 34.43 microplastic/individual. The highest microplastic count was 1047 microplastic/individual (DL 58.72 mm, DH 50.96 mm) and the lowest microplastic count was 12 microplastic/individual (DL 76.14 mm, DH 63.56 mm).

The skewness of size class (0-49.9 mm) was 2.035 and skewness of size class (50.0-99.9 mm) was 2.805. Both size classes (0-49.9 mm) and (50-99.9 mm) showed positive

skew, therefore the data set was not close to normal distribution. The data sets were then tested for normality (Shapiro-Wilk test) for confirmation and selection of statistical analysis type.

Shapiro-Wilk tests were done for both sets of data (class size 0-49.9mm) and (class size 50-99.9 mm). This aim of the test is determining normality of the data sets and selection of suitable statistical test (non-parametric or parametric test) for further analysis. The null hypothesis for the current Shapiro-Wilk test is that the tested data set is normally distributed. The level of significance selected for the current Shapiro-Wilk test is $\alpha=0.05$. The result of the Shapiro-Wilk test was shown in Table 4.2.

Table 4.2 Shapiro-Wilk test for size class to microplastic ingestion data sets. (Retrieved from SPSS 28.0)

		Kolmogorov-Smirnov ^a			Shapiro-Wilk		
	Size (mm)	Statistic	df	Sig.	Statistic	df	Sig.
Microplastic ingestion	0-49.9	0.180	46	<0.001	0.805	46	<0.001
	50.0-99.9	0.225	35	<0.001	0.689	35	<0.001

A Shapiro-Wilk test showed a significant departure from normality, $W(46) = 0.805$, $p = <0.001$.

A Shapiro-Wilk test showed a significant departure from normality, $W(35) = 0.689$, $p = <0.001$.

Null hypothesis of both tested data sets (0-49.9 mm) and (50.0-99.9 mm) were rejected as the p value is < 0.05 , therefore a non-parametric statistical analysis, Mann-Whitney U test was selected to test the differences in microplastic ingestion from different class size (0-49.9 mm) and (50-99.9 mm).

Result of Mann-Whitney U test of microplastic ingestion between two different size classes (0-49.99 mm) and size class (50.0-99.99 mm) were shown in Table 4.3.

Table 4.3 Mann-Whitney U test result of size class (0-49.9mm) and (50-99.9mm) to microplastic ingestion. (Retrieved from SPSS 28.0)

Ranks				
	Size (mm)	N	Mean rank	Sum of ranks
Microplastic ingestion	0-49.9	46	46.48	2138.00
	50-99.9	35	33.80	1183.00
	Total	81		
Mann-Whitney U		553.00		
Asymptotic Sig (2-sided test)		0.016		

The p value is < 0.05, therefore the result showed that there was a significant difference (U=553, p=0.016) between microplastic ingestion for size class (0-49.99 mm) and size class (50.0-99.99 mm). The median for size class (0-49.9 mm) was 150.50 microplastic/individual⁻¹ compared to 87.0 microplastic/individual⁻¹ for size class (50.0-99.9 mm). The results indicated that the smaller size class (0-49.9 mm) of *M. lyrata* have more microplastic ingestion compared to the bigger size class (50.0-99.9mm) of *M. lyrata*.

4.2.2 Microplastic ingestion according to factor: harvesting season

The samples were rearranged according to each representative harvesting season (pre monsoon and post monsoon). There are a total of 36 samples in group per monsoon and 45 samples in group post monsoon. Figure 4.9 shows the total amount of microplastic ingestion sorted according to the clam size and harvesting seasons. Descriptive analysis between

harvesting season pre-monsoon and post-monsoon to microplastic ingestion shown in Table 4.4.

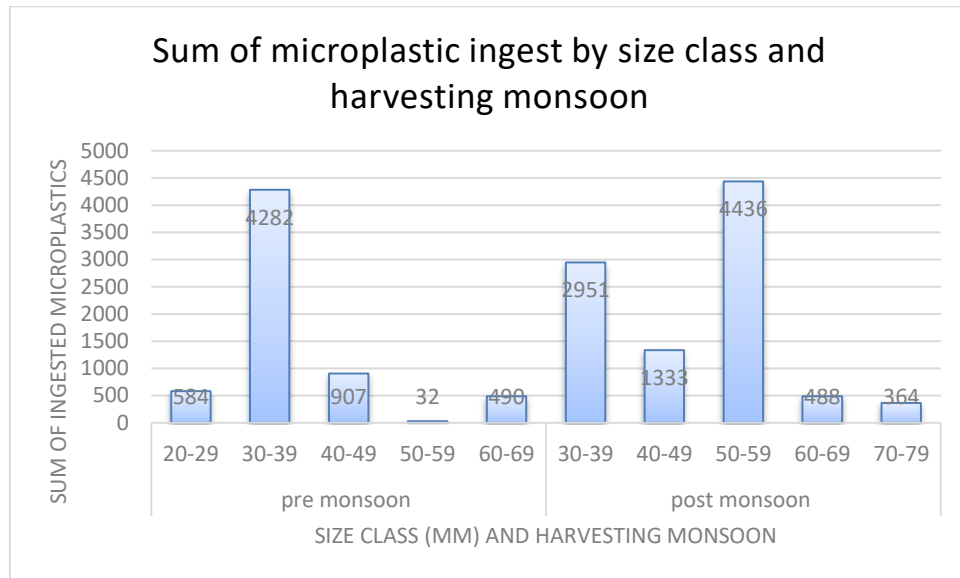


Figure 4.9 Sum of microplastic ingested sorted according to size class and harvesting season of *M. lyrata*.

Table 4.4 Descriptive analysis result for harvesting season (pre-monsoon) and (post-monsoon) to microplastic ingestion (mp) (Retrieved from SPSS 28.0)

Descriptive				
Harvesting season		Statistic		Standard Error
Pre-Monsoon	Sample size	36		
	Mean	174.8611		19.60779
	95% Confidence interval for mean	(Lower Bound) = 135.0552 (Upper Bound) = 214.6670		
	5% Trimmed Mean	166.0988		
	Median	144.5000		
	Variance	13840.752		
	Standard deviation	117.64672		
	Skewness	0.960		0.393
	Kurtosis	0.602		0.768
Microplastic ingested	Post-Monsoon	Sample size	45	
	Mean	212.7111		35.57566
	95% Confidence interval for mean	Lower Bound = 141.0131 Upper Bound = 284.4092		
	5% Trimmed Mean	181.2284		
	Median	129.0000		
	Variance	56953.256		
	Standard deviation	238.64881		
	Skewness	2.018		0.354
	Kurtosis	4.208		0.695

The mean value for pre-monsoon was 174.86 ± 19.61 microplasticindividual⁻¹. The highest microplastic count in harvesting season (pre monsoon) was 507 microplastic/individual⁻¹ (DL 36.26 mm, DH 31.46 mm) and the lowest count was 28 microplastic/individual⁻¹ (DL 60.64 mm, DH 52.00 mm).

The mean value for post monsoon was 212.71 ± 35.58 microplasticindividual⁻¹. The highest microplastic count in harvesting season (post monsoon) was 1047 microplastic/individual⁻¹ (DL 58.72 mm, DH 50.96 mm) and the lowest microplastic count was 12 microplastic/individual⁻¹ (DL 76.14 mm, DH 63.56 mm).

The skewness of the harvesting season (pre-monsoon) was 0.96 which shows a moderate skew. The skewness of the harvesting season (post-monsoon) was 2.018 which shows a highly skewed distribution. Both harvesting season (pre monsoon) and (post monsoon) shows positive skew distribution, therefore the data sets were not close to normal distribution. The data sets were then tested for normality (Shapiro-Wilk test) for confirmation and selection of statistical analysis type.

Shapiro-Wilk tests were done for both sets of data (pre monsoon) and (post monsoon). This aim of the test is determining normality of the data sets and selection of suitable statistical test (non-parametric or parametric test) for further analysis. The null hypothesis for the current Shapiro-Wilk test is that the tested data set is normally distributed. The level of significance selected for the current Shapiro-Wilk test is $\alpha=0.05$. The result of the Shapiro-Wilk test was shown in Table 4.5.

Table 4.5 Shapiro-Wilk test for harvesting season to microplastic ingestion data sets.

	Harvesting season	Kolmogorov-Smirnov ^a			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Microplastic ingestion	Pre monsoon	0.154	36	0.031	0.919	36	0.012
	Post monsoon	0.200	45	<0.001	0.754	45	<0.001

A Shapiro-Wilk test showed a significant departure from normality, $W(36) = 0.919$, $p = 0.012$.

A Shapiro-Wilk test showed a significant departure from normality, $W(45) = 0.754$, $p = <0.001$.

Null hypothesis of both tested data sets (pre monsoon) and (post monsoon) were rejected as the p value is < 0.05 , therefore a non-parametric statistical analysis, Mann-

Whitney U test was selected to test the differences in microplastic ingestion from different harvesting season (pre-monsoon) and (post-monsoon).

Results of Mann-Whitney U test between different harvesting seasons (pre-monsoon) and (post-monsoon) were shown in Table 4.6.

Table 4.6 Mann-Whitney U test result for harvesting season (pre-monsoon) and (post monsoon) to microplastic ingestion.

		Ranks		
	Season	N	Mean rank	Sum of ranks
Microplastic ingestion	Pre-monsoon	36	42.53	1531.00
	Post-monsoon	45	39.78	1790.00
	Total	81		
Mann-Whitney U		755.00		
Asymptotic Sig (2-sided test)		0.601		

The p value is > 0.05 , therefore there was no significant difference ($U=755$, $p=0.601$) between microplastic ingestion for harvesting season (pre monsoon) and (post monsoon). The median for pre monsoon was 144.50 microplastic/individual⁻¹ compared to 129.00 microplastic/individual⁻¹. There are no significant differences in microplastic ingestion under the factor of harvesting season.

4.3 Polymer identification using FTIR

The microplastic items isolated for polymer identification were limited to items which are visible to the naked eye. Summary of isolated suspected particles based on shape were shown in Table 4.7.

Table 4.7 Shape classification of isolated suspected particles for FTIR-ATR analysis.

Shape classification of sample	Count
Fibre	2
Fragment	17
Particle	1

Samples categorized as particles were excluded from isolation due to its small size and high tendency of false results throughout the identification process. 85 % of the samples categorized as fragments were isolated for FTIR analysis followed by 2 fibers and 1 particle. The selected particle type was suspected to be fragmented from bigger pieces therefore selected for analysis. 1 particle shape is chosen because this is one of the bigger and visible sizes among other detected particles.

A total of 20 samples of IR spectrum were retrieved from Nicolet IN10 and compared with two different reference libraries, OMINIC software in-built library and open-source reference library (OpenSpecy). Table 4.8 shows the comparison of spectral data with two different reference libraries (OMINIC software in-built library) and (OpenSpecy) an open-source reference library.

Table 4.8 Comparison of spectral data with two different reference libraries (OMINIC software in-built library) and (OpenSpecy) an open-source reference library according the spectral match pair

Sample	OMINIC Nicolet IN10	OpenSpecy
1.1	Cellophane (0.55)	Polyvinylchloride (0.76)

3.1	Cellophane (0.58)	Cellulose (0.78)
3.2	Rubidium nitrate (0.73)	Polyvinylchloride (0.72)
4.1	Polyethylene white layer (TiO ₂) (0.60)	Polyvinylchloride (0.75)
4.3	Cellophane (0.57)	Polyvinylchloride (0.75)
6.2	Bentonite (0.74)	Cellulose (0.85)
6.3 a	Cellophane (0.64)	Cellulose (0.86)
6.3 b	Cellophane (0.58)	Polyvinylchloride (0.75)
8.1	Cellophane (0.58)	Cellulose (0.81)
10.3 a	Sodium polyphosphate (0.40)	Cellulose (0.85)
10.3 b	Cellophane (0.66)	Polyvinylchloride (0.75)
14.1	Cellophane (0.61)	Polyvinylchloride (0.75)
1.2 bottom a	Cellophane (0.58)	Chitin from crustacean shells (0.82)
1.2 bottom b	Silopren E 3078 (0.67)	PDMS (0.83)

1.2 bottom c	Cellophane (0.57)	PDMS (0.79)
post 2.1	poly(ethylene:propylene:diene) (0.48)	Polyvinylchloride (0.84)
post 1.2	Cellophane (0.58)	Chitin from crustacean shells (0.91)
post 2.3	Cellophane (0.53)	Cellulose (0.84)
Clam trial sample 1	Bentonite (0.57)	Resin dispersion (0.85)
Clam trial samples 2	Bentonite (0.51)	Polypropylene with silicate mix (0.82)

75 % of the items were classified as plastics according to the OMINIC software in-built library whereas only 60 % of the items were identified as plastics when compared with OpenSpecy database. Nicolet IN10 library identified 7 types whereas OpenSpecy identified 6 different types of polymers. It is to be noted that only two items identified using Nicolet IN10 database have more than 70 % match on the other hand, all items identified using OpenSpecy database have more than 70 % match with reference library data. OpenSpecy database is selected due to the higher match percentage compared to Nicolet IN10 database.

The most common plastic polymer identified was polyvinyl chloride (40 %), followed by PDMS (10 %), resin dispersion (5 %) and polypropylene with silicate mix (5 %). A total of 8 particles were identified as non-plastic polymer, cellulose (30 %) and chitin from crustacean shells (10 %). Figure 4.10 shows a summary of composition of identified polymers using a pie chart.

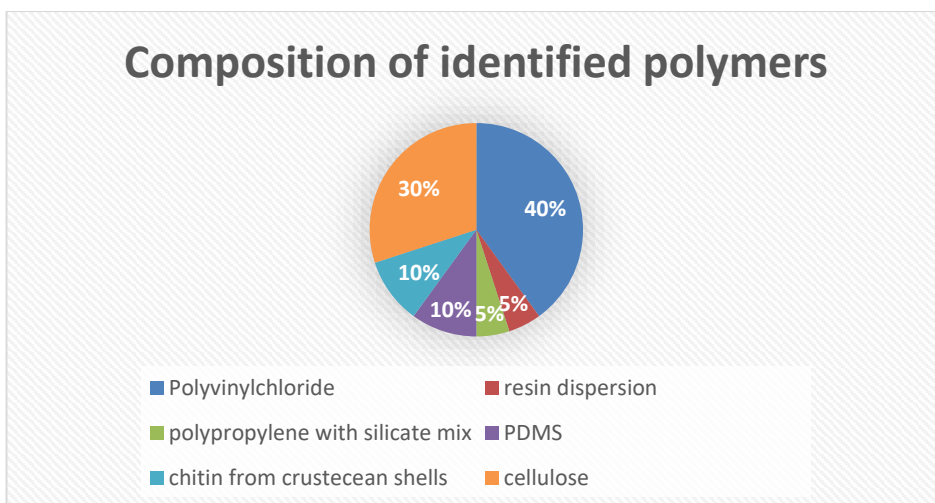


Figure 4.10 Composition of identified polymers from the suspected particles.

Types of identified polymers sorted according to size class and harvesting seasons were shown in Figure 4.11. Both cellulose and polyvinyl chloride were the two polymers which were observed in all size classes (30-39, 40-49, 50-59 mm) and harvesting season (pre monsoon and post monsoon). An interesting observation was observed from the result, all types of identified polymer were detected from particles isolated from class size 30-39 mm. Two unique polymers, resin dispersion and PDMS were identified from the same class size (30-39 mm) but different harvesting season, resin dispersion in pre monsoon samples and PDMS in post monsoon samples. Chitin from crustacean shells were observed in particles isolated from size class (30-39 and 50-59 mm) and in post monsoon. Polypropylene with silicate mix was observed in particles isolated from clam (40-49 mm, pre monsoon).

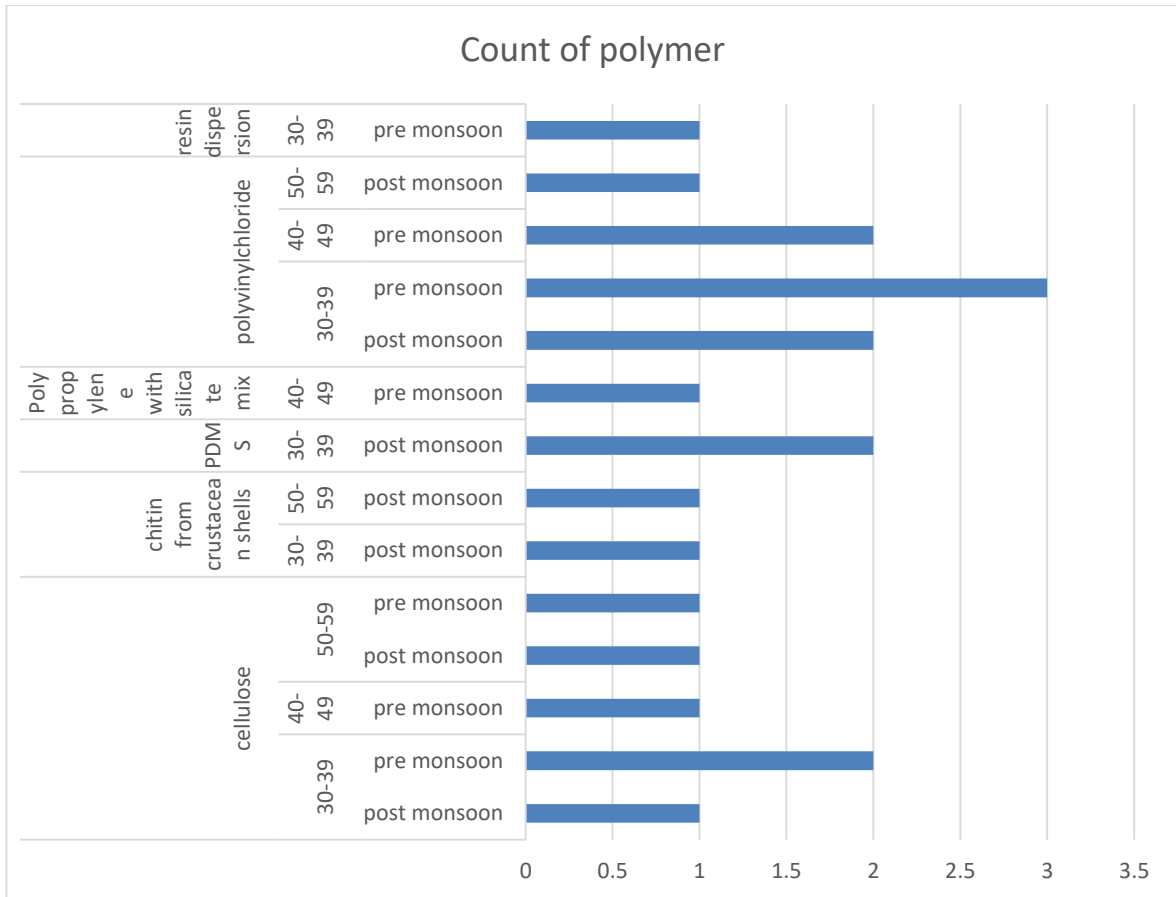


Figure 4.11 Identified polymer sorted according to size class and harvesting monsoon. Overlapping of spectrum from isolated particles with standard spectrum were done using OpenSpecy. The spectrum comparison result of the six identified plastic polymers with an open-source database (OpenSpecy) were shown in Figure 4.12.

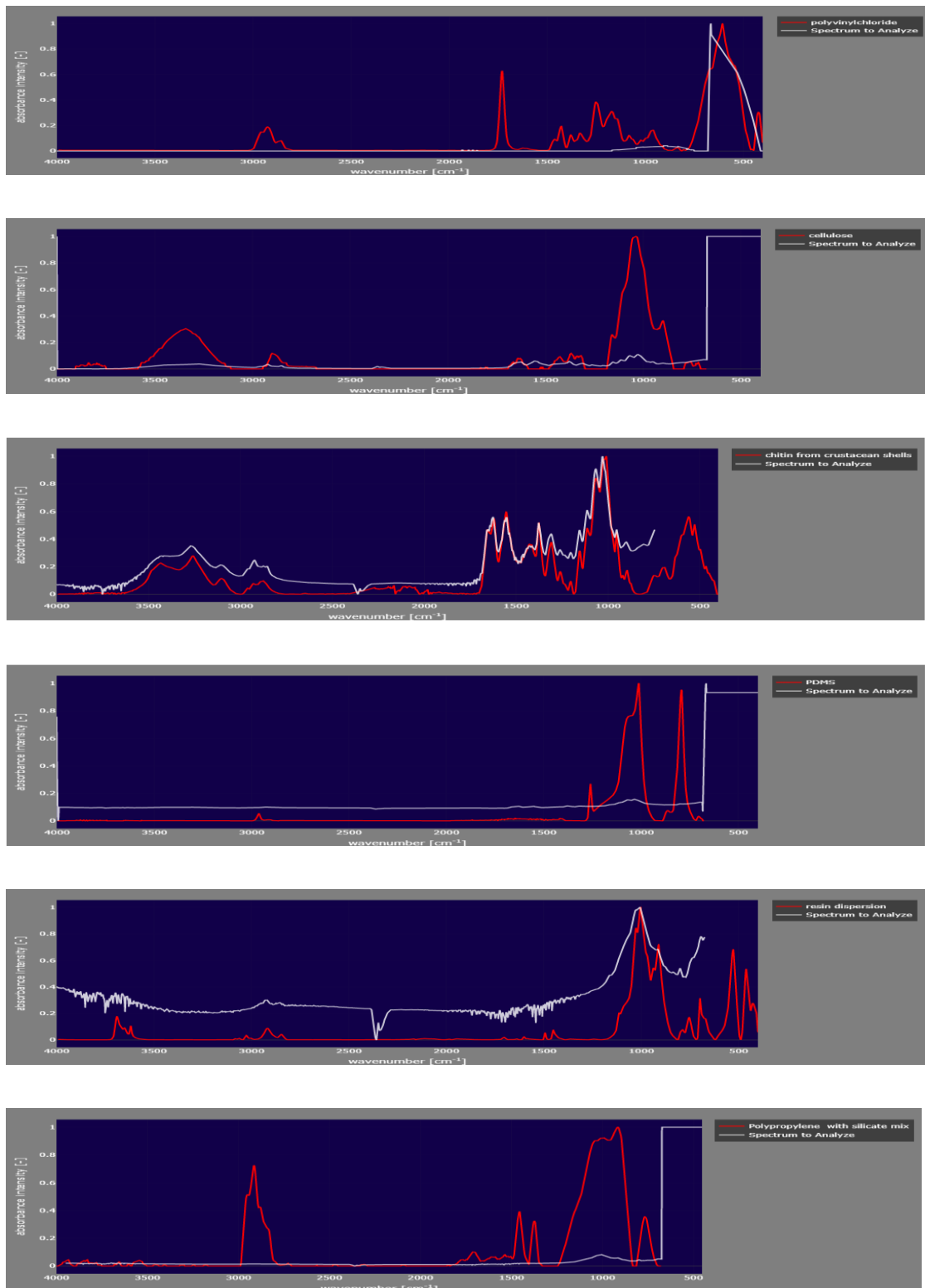


Figure 4.12 Spectrum comparison result of the six identified plastic polymers with an open-source database (OpenSpecy).

The overlapping of spectrum in which the red spectrum represents the standard spectrum in the database having the closest similarity in spectrum peaks after comparing with the spectrum to be analyzed. All spectrums were generated from FTIR IN10 with the unit of transmittance (%), the spectrum will be auto corrected to the unit of absorbance intensity when using OpenSpecy database.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

Fluorescent tagging is a new method which can increase the detection sensitivity of microplastic quantification through visual detection. The combination of different methods to the existing quantification method requires adaptations. In this project, two additional steps were added to combine alkaline digestion (KOH) with density separation (ZnCl_2) and fluorescent tagging using Nile red.

The first adaptation is adding an acidified step upon alkaline digestion (10% KOH) reaching the endpoint. This is to prevent the formation of ZnOH_2 (white precipitation) when direct mixing alkaline digestion solution with density separation medium (ZnCl_2). The formation of white precipitate can also be caused by reaction between ZnCl_2 with water. It was observed that the precipitation formation can be reduced through lowering pH, therefore it's crucial to acidify the digestion solution but not neutralize. 10% HCl is chosen as the acid solution for acidification because it will give out same negative ion (Cl^-) therefore less interference towards density separation process.

The second adaptation is adding a rinsing step using 10% HCl and followed by rinsing with filtered distilled water before and after performing fluorescent tagging protocol. This is to dissolve the potential small precipitation occurring during density separation. The removal of precipitation is important because it can reduce the sensitivity of fluorescent tagging through masking the microplastic particles with the precipitation which prevents the fluorescent tagging using Nile red.

The average microplastic quantified from the current project was 195.90 ± 43.6 microplastic/individual. This finding is a lot higher than the other findings from nearby countries with same species (*M. lyrata*) 12.73 ± 4.49 microplastic/individual, 13.20 ± 7.66 microplastic/individual (Tran-Nguyen et al., 2023), 3.6 ± 2.1 microplastic/individual (Kieu-Le et al., 2022) and 0.67 ± 0.15 microplastic/individual. (Chinfak et al., 2021).

The average dimension of microplastics from the current project is between 0.008 mm - 2.82 mm. Current project dimension range of detected microplastic is lower than the other findings 0.3 mm - 4.5 mm (Tran-Nguyen et al., 2023) ,0.3 mm - 5.0 mm (Kieu-Le et al., 2022) and 0.24 mm - 2.253 mm (Chinfak et al., 2021). Moreover the most detected microplastic shape for the current project is particle size. This is different from other findings within Malaysia (species ranging from *Corbivula fluminea*, *Polymesoda expansa*, *Paratapes undulatus*, *Perna viridis*) which fiber and fragment were reported as the most abundant microplastic shape detected in shellfish. (Mazlan et al., 2022; Mazni et al., 2022; Karim et al., 2023). Similar results were reported from the findings related to the shape of microplastic detected in shellfish where fiber was claim the most abundant microplastic shape within shellfish (species ranging from *Meretrix lyrata* ,*Paratapes undulatus*,) (Chinfak et al., 2021; Kieu-Le et al., 2022; Tran-Nguyen et al., 2023).

These differences might be due to the sensitivity of the detection method chosen. Kieu-Le et al. (2022) and Tran-Nguyen et al. (2023) used 10% KOH for alkaline digestion protocol but only Tran-Nguyen et al. (2023) performed density separation using NaCl solution (1.18 g cm^{-3}). Using denser medium for density separation can increase the sensitivity of microplastic detection. Quinn et al. (2017) concluded using denser mediums to perform density separation have higher recovery rates compared to lower density

mediums such as water (1 g cm^{-3}) and saturated NaCl ($\sim 1.18 \text{ g cm}^{-3}$). Zarlf (2019) also reported extracting lower density microplastic using saturated NaCl may also fail if the plastic particles are agglomerated with mineral particles. Chinfak et al. (2021) used oxidative digestion using 30% H_2O_2 and density separation using saturated NaCl. Nuelle et al. (2014) advised the usage of oxidative digestion method should be only applied to organic rich samples because of the bleaching effect that might aggravate the visual detection process and cause potential exothermic reaction with other chemicals (NaI, for density separation).

The fluorescent tagging protocol on the other hand is an additional step which can reduce the workload and increase the sensitivity of microplastic detection. With proper purification steps done to remove other potential materials (lipids and chitin), fluorescent tagging using Nile red can show fluorescence of the ‘tagged’ microplastics under favorable conditions (blue light emission and viewed with an orange filter). This may be one of the reasons for different detection of microplastic concentration compared to findings targeting *M. lyrata* from other researchers.

The results from microplastic ingestion were sorted according to the size class. The size class was then further reclassified into two groups: (0-49.99 mm) and (50.0-99.99 mm). The justification for the size class was that the smaller size class was the most abundant size range of *M. lyrata* in imported packed *M. lyrata* (refer Appendix 4) or those sold in local fish markets. The result shows the smaller size class had significant higher microplastic detection compared to the bigger size class group. The microplastic ingestion in smaller sized class clam (0-49.9 mm) with the average of 218.63 ± 54.78 microplastic/individual compared to size class (50.0-99.9 mm) with the average of 166 ± 68.86 microplastic/individual. Similar results were reported by Chinfak et al. (2021), smaller size

M. edulis and *M. lyrata* have significantly higher microplastic ingestion compared to the larger counterparts. Scott et al. (2019) and Sathish et al. (2020) also reported similar microplastic detection patterns in *M. edulis* and *Donax cuneatus*. Winter (1973) and Sylverter et al. (2005) reported decrease in larger bivalve filtration rate and this may prevent ingestion of more microplastic per weight compared to their smaller counterparts. Sylverter et al. (2005) explained as bivalves age, the gill surface to body size ratio is reduced, whereas filtration is high in the smaller bivalve due to the higher metabolic demand in the younger individual. Han et al. (2007) also reported a 28% filtration difference between the smaller and bigger size clams, in which the smaller clams have higher filtration rate compared to the bigger clams. Woods et al. (2018) reported certain bivalves are selective filter feeders in which they have the ability to reject some of the ingested particles such as microplastic through excretion. The capacity of ingestion, accumulation and egestion may differ according to habitat, Ward et al. (2019) further explained that this is also dependent on the surface characteristics, concentration and the size of microplastic.

Statistical result from current project shows that there is no significant difference between harvesting season (pre-monsoon and post-monsoon), for which pre-monsoon (174.86 ± 21.2 microplastic/individual) and post-monsoon (212.71 ± 71.14 microplastic/individual). The possible microplastic input for the study site is through both land input (human settlement area) and input through river systems (oil palm plantation, aquaculture farm) and input through tidal change and wave activities. Mobilik et al. (2013; 2014) did an analysis on quantification of marine debris from selected beaches in Sarawak during different monsoon seasons. The result from their findings shows their study site Pasir Pandak beach (similar geomorphology and populated fishing village) recorded more marine debris collected during the Northeast monsoon (1120 items/km) compared to marine debris

collected during Southwest monsoon (584 items/km). Similar observations were expected from the current study site (Kampung Buntal) as both sites receive direct impact from tidal action and man-made protection was built to protect the coastline from further erosion.

The result for the current project shows that the availability of microplastic was similar between the two monsoons. Lebraton et al. (2019) hypothesized that most of the plastic marine debris should be settling in the coastal environment through a series of processes such as stranding, settling and resurfacing process. The mentioned process can also break down the bigger plastic marine debris into smaller microplastics (through physical abrasion), therefore the plastic marine debris and the byproduct (microplastics) will be highly polluting the habitat of the *M. lyrata* (bivalve harvesting site visualize in Figure 3.1). Higher microplastic abundance in surface sediment of intertidal zone during neap tide cycles compared to spring tide cycles as reported by Wu et al. (2020). The microplastic settled at surface sediment during the neap tide cycle can either be brought away by strong tidal actions during spring tide cycles or buried in the sediment column. *M. lyrata* as one of the common bivalves inhabits at intertidal zone were highly at risk of microplastic contamination due to the high bioavailability of microplastic within the region.

Polymer identification using Nicolet IN10 was done on randomly 20 isolated suspected particles. 6 polymers were identified in which 4 was plastic polymers (polyvinyl chloride (PVC)- 8 samples, polydimethylsiloxane (PDMS)- 2 samples, resin dispersion- 1 sample, and polypropylene with silicate mix (PP with silicate mxi)- 1 sample) and 2 polymers was non plastic polymers (cellulose- 6 samples and chitin from crustacean shells- 2 samples). Local publications also reported similar detection of plastic polymer, polyvinyl

chloride and polypropylene in both *Corbicula fluminea* and *Polymesoda expansa* (Karim et al., 2023) and polypropylene in *Perna veridis* (Mazni et al., 2022).

Karim et al. (2023) mentioned a lower absorbance from the polymers isolated from commercial bivalves than reference polymer spectra. Jambeck et al. (2015) explains the decrease in the intensity peak may correspond to the photo-degeneration process. Löder & Gerdtz (2015) explains environmental samples may undergo degradation processes and biofilms with combination of polymer additives may increase the difficulties of spectroscopic analysis causing spectral changes.

Similar observations were observed in the current project, therefore a comparison with two different reference libraries was done and to select the better spectra match database. Only the results of spectral match higher than 70% is acceptable (Su et al., 2020), therefore the results from comparing with ‘OpenSpecy’ database were chosen and accepted as polymer identification results of the current study.

All 4 types of detected plastic polymers (PVC, PDMS, PP and resin) were either common additives or building materials and easily entered the environment if there were human settlements around the area.

Polyvinyl chloride (PVC) is commonly used in the building and construction sector. It is a thermoplastic having a good resistance to alkalis, salt and highly polar solvents. Despite the relatively high density of PVC in nature, formation of biofilm may also alter the density through increasing the density and decreasing the buoyancy of the particles. (Rummel et al., 2017).

Polydimethylsiloxane (PDMS) is an elastomeric polymer used as fuel additives in car waxes, cleaners and polishers, and biomedical application such as implants for cosmetic surgery. One of the routes that PDMS can enter the aquatic environment is through wastewater treatment plants or directly introduced into the aquatic environment as wastewater (Álvarez-Muñoz et al., 2016). PDMS adsorb strongly to sludge which reduces the volatility of PDMS (Whelam et al., 2009). The combination of PDMS with sludge will settle down in the environment, therefore PDMS is usually found in high concentration near the wastewater discharge areas (Sparham et al., 2011).

Polypropylene (PP with silicate mix) is one of the common plastics used in human daily life. Single use plastic bags are a good example of polypropylene products. Fishing lines and nets were mostly composite of PP and PE. Improper discard of broken nets may break down into smaller pieces and form microplastics.

Resin dispersion according to Yoshida (2014) is formed from epoxy resin after mixing with different component such as basic epoxy resin, curing agents, accelerators and additives to produce epoxy with specific performance, therefore it's hard to determine the density of the dispersed resin. Current fishing boats materials are shifting from wood to glass fiber boats due to its durability and lightweight. Resins were commonly used as the main adhesion agent in repairing fiber boats. This might explain the availability of this polymer within the study site.

Findings from Ke et al. (2019) and Luan et al. (2019) shows the exposure of microplastics (polypropylene and polystyrene) to clams (*Meretrix meretrix*) can lead to reduction in total oocyte number, oocyte diameter peroxidation damage of embryo membrane and death of *M. meretrix* larvae when rearing with microplastic exposure.

The possibility of microplastic entering humans is possible through consumption of seafood such as bivalves because bivalves were usually consumed as whole organisms rarely with gut removal. (Cho et al., 2019). The toxicity of the plastic additives and chemicals adhered to the surface of microplastic during exposure in the environment may affect humans upon consumption of bivalves or other seafood that are contaminated with microplastics. (Karbalaee et al., 2018).

5.2 Conclusion

All samples were found contaminated with microplastic (100% microplastic detection). The average microplastic detection was 195.90 ± 43.6 microplastic/individual. The most quantified shape of microplastic was particle shape. This is different from other local and international publications which suggested fiber as the most abundant microplastic shape. The high detection (89%) of particle shape microplastic might be due to the high sensitivity method design for microplastic quantification such as combining two purification steps (alkaline digestion and density separation) and fluorescent tagging method using Nile red. Proper removal of non plastic substances is important in microplastic detection and fluorescent tagging using Nile red can aid in rapid screening in both quantification and isolation for polymer identification.

Current study suggested a few adaptation steps to improve existing microplastic quantification methodology such as acidify digested matrix with 10 % HCl and rinsing of excess Nile red after 10 minutes incubation period. The statistical results (smaller clams have higher microplastic ingestion but no significant difference between harvesting seasons) show that the study site is highly polluted with microplastics and smaller size clams were having

more microplastic ingestion compared to the bigger size clam as the filtration rate is higher in the smaller size clam.

The identified plastic polymers from the polymer identification are PVC, PDMS, resin and PP with silicate mix. High spectral match result is highly dependent on the constant update of existing reference libraries. Environmental samples can hardly have spectral match near to 100% due to the contamination or degradation from UV or abrasion.

High microplastic ingestion of *M. lyrata* is a worrying issue as it is one of the local preferred shellfish species. The habit of consuming the clam as a whole increases the risk of microplastic entering the human diet. Depuration method may be a good method to reduce the microplastic content inside of shellfish. It is advisable to perform depuration in salt water (~ 35‰ salinity) on the shellfish prior to consuming the shellfish.

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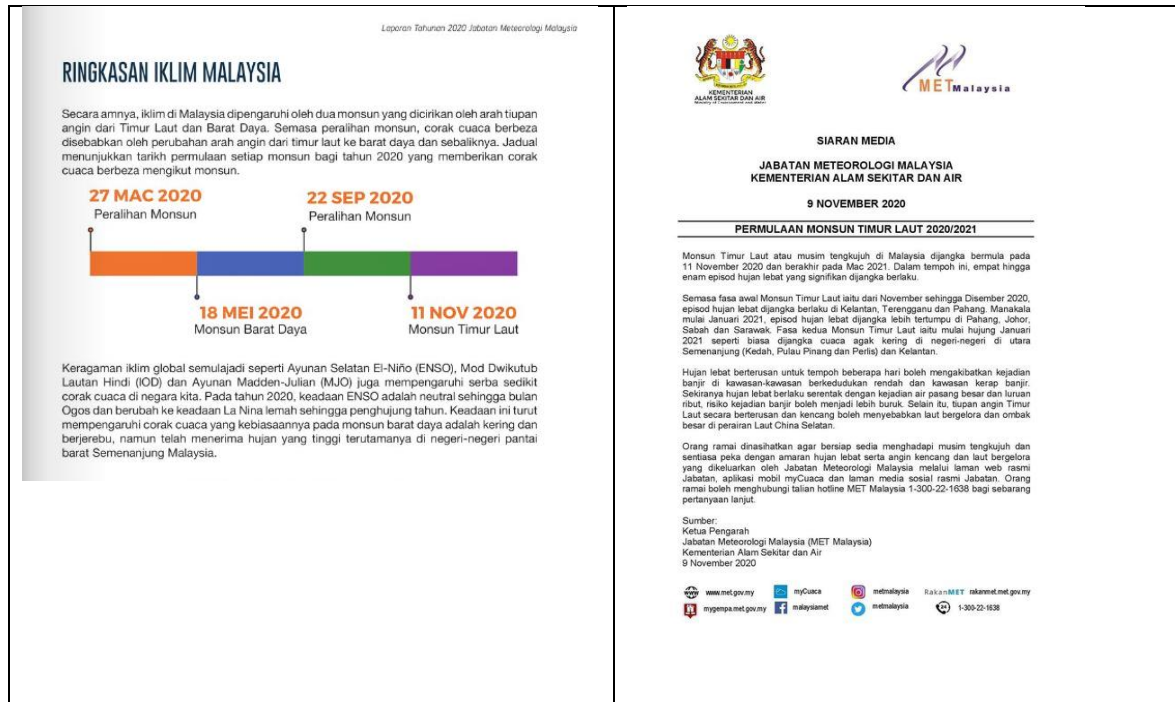
APPENDICES

Appendix 1



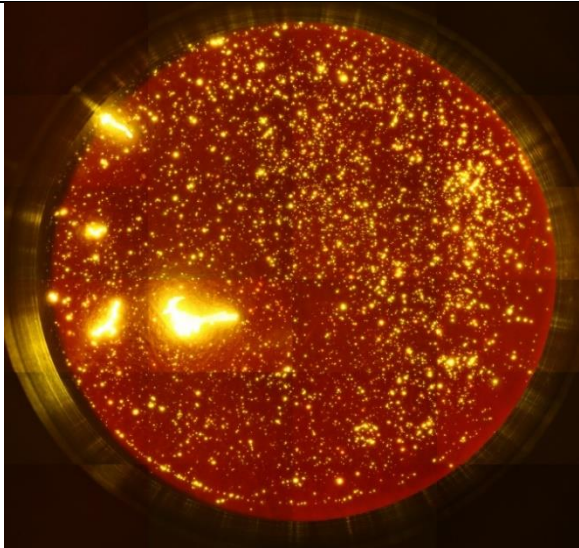
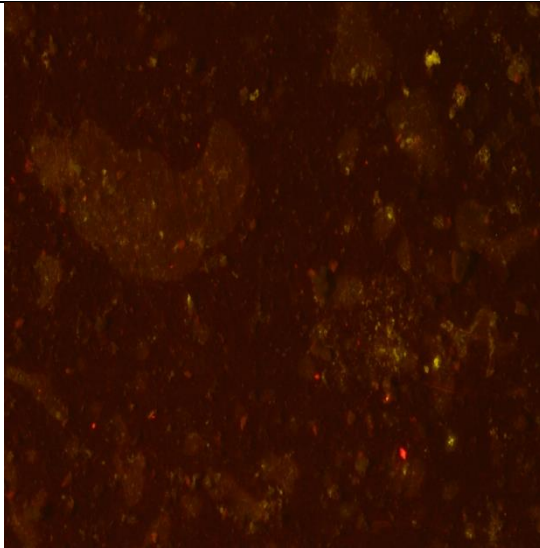
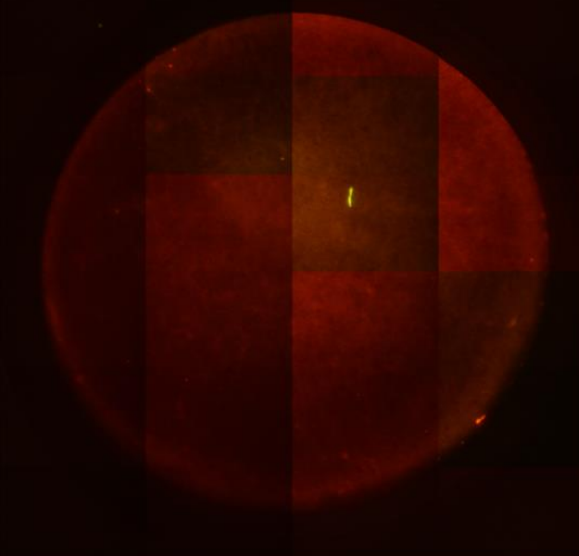
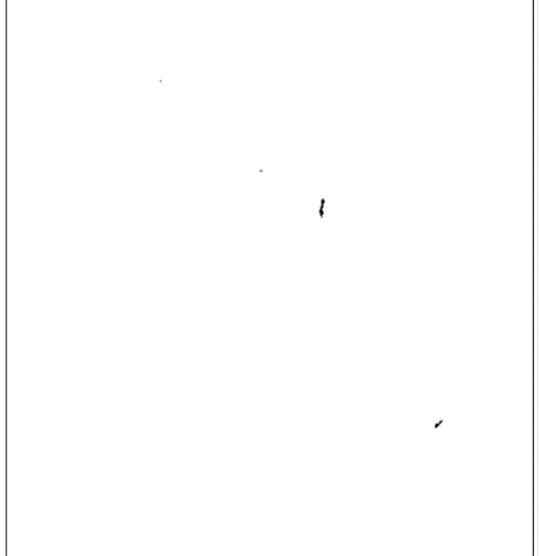
Automated filter-scanning rig

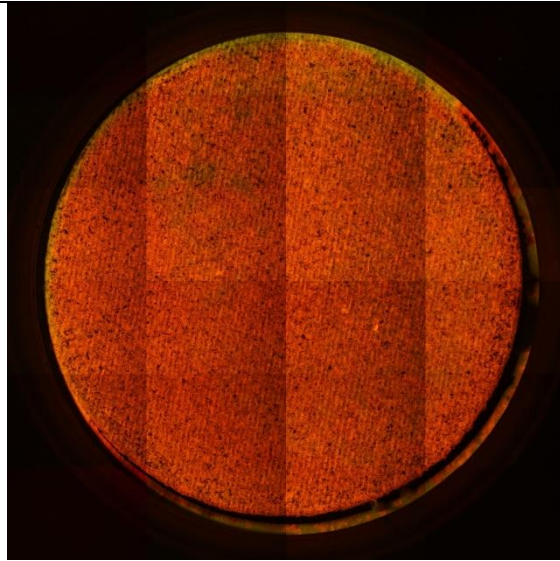
Appendix 2



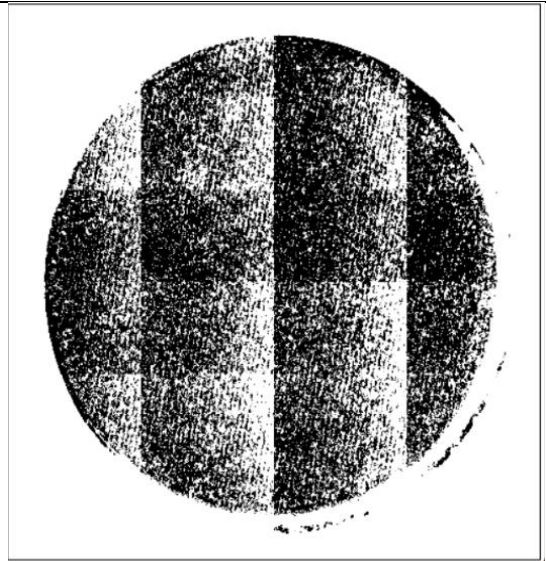
Dates of pre-monsoon and post-monsoon obtained from Metrology Malaysia.

Appendix 3

	
Interference observed without HCl rinsing protocol	Possible 'masking' by precipitation and reduce the staining efficiency
	
A perfect example, clear fluorescence and no interference from non plastic particles.	After processed into 8 bits by MP-VAT for automated quantification



Smearing of Nile red without removal of excess Nile red dye through rinsing



MP-VAT failed to identify between stained particles and unstained dye.



Fragment particles identified as PDMS

Appendix 4



Imported white clam with size class between 0-49.99 mm

Appendix 5



Class size (50.0-99.9mm) clam example (DL:55.36mm, DH: 47.30mm)

Appendix 6

Species	Location	Microplastic ingested (items/individual)	Reference
<i>Meretrix lyrata</i>	Danang, Vietnam	12.73 ± 4.49	Tran-Nguyen et al., 2023
<i>Meretrix lyrata</i>	Danang, Vietnam	13.20 ± 7.66	Tran-Nguyen et al., 2023
<i>Meretrix lyrata</i>	Ho Chi Minh, Vietnam	3.6 ± 2.1	Kieu-Le et al., 2022
<i>Meretrix lyrata</i>	Surat Thani, Thailand	0.67 ± 0.15 ~ 0.23 ± 0.09	Chinfak et al., 2021
<i>Paratapes undulatus</i>	Danang, Vietnam	3.43 ± 0.98	Tran-Nguyen et al., 2023
<i>Paratapes undulatus</i>	Danang, Vietnam	3.30 ± 0.94	Tran-Nguyen et al., 2023
<i>Tapes philippinarum</i>	South Korea	1.15 ± 0.74	Cho et al., 2019
<i>Perna viridis</i>	Johor, Malaysia	2.23 ± 1.04	Mazni et al., 2022
<i>Perna viridis</i>	Johor, Malaysia	1.29 ± 1.19	Mazni et al., 2022
<i>Perna viridis</i>	Thanh Hoa, Vietnam	2.60 ± 1.14	Nam et al., 2019
<i>Mytilus edulis</i>	South Korea	0.68 ± 0.64	Cho et al., 2019
<i>Mytilus galloprovincialis</i>	Qingdao, China	0.53	Jin-Feng et al., 2018
<i>Mytilus galloprovincialis</i>	Qingdao, China	1.9	Jin-Feng et al., 2018
<i>Corbicula fluminea</i>	Kelantan, Malaysia	14.7 ± 7.07	Karim et al., 2023
<i>Polymesoda expansa</i>	Kelantan, Malaysia	1.32 ± 0.7	Karim et al., 2023

List of publication of microplastic ingestion of bivalves.