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Research on Microplastics in Marine Life in the Coastal Areas of Japan

（日本近海におけるマリンライフ中の
マイクロプラスチックスの研究）

Hokkaido University　Graduate School of Global Food Resources Division of Global Food Resources　Doctoral Course

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Contents

Chapter 1: Introduction

1.1 Marine pollution

Oceans cover over 70% of the earth's surface. Millions of creatures exist, from microscopic plankton to giant whales [1]. Oceans also significantly impact the global climate system by regulating temperature, weather patterns, and the carbon cycle [2].

Marine pollution is a major global environmental concern because of the significant risks. It can harm the health and well-being of humans and marine ecosystems [3]. This affects all levels, from low-level microbes to apex predators, through various pathways that harm the entire marine ecosystem. Impacts on sediments, eutrophication, toxic substances, and marine debris are reported as marine pollution. Various pollution causes include intensive agriculture, urbanization, industrialization, deforestation, and coastal developments [4]. It occurs when harmful substances or materials are intentionally or unintentionally introduced into the ocean [5]. Chemical pollutants such as oil, plastic, and heavy metals, and biological contaminants such as pathogens and invasive species can be listed among these harmful substances [6]. Marine pollution harms marine ecosystems in several ways, including habitat destruction, species extinction, and ecosystem degradation [7]. These pollutants include chemical compounds, petroleum-derived substances, marine debris, sewage-associated pathogens, and excess nutrients with high environmental impacts [8].

Plastics, including microplastics and larger plastic debris (macro-plastics), are a big issue. Plastic is a durable and versatile material used in various products, such as packaging, toys, and construction materials [9], with much of it ending in the ocean [10]. Macro-plastics can lead to entanglement and suffocation in birds, fish, and sea turtles [11]. Moore [12] found that approximately 60% of sea turtles have consumed plastic.

Moreover, microplastics can be hazardous to marine animals and human health and transport harmful chemicals and pathogens [13] into the food chain and end up in the seafood we eat [14].

Sustainable Development Goal 14 aims to “Conserve and sustainably use the oceans, seas and marine resources for sustainable development.” In order to achieve this goal, efforts from all sectors of society are required, including increased research on marine life [15]. Marine mammals are at the top of the marine food chain and can accumulate some of the highest levels of environmental pollutants of all wildlife [16]. Given the increasing prevalence and severity of marine wildlife diseases, it is important to understand the types and mechanisms of the toxicity of pollutants. For example, persistent pollutants have been associated with various toxic effects in marine mammals, including immunosuppression and the development of infectious diseases [17]. Considering the background knowledge relevant to this study, this chapter focuses on the impacts of microplastics in the marine ecosystem and summarises the research on microplastics and their importance for the main objective of this thesis.

1.2 Plastic pollution

Plastic pollution is a major environmental concern that has garnered significant attention in recent years. Plastic pollution is defined as the presence of plastic materials in the environment with adverse effects on wildlife, habitats, and human populations [18]. Marine litter (marine waste, garbage, or debris) is every type of man-made solid waste that deliberately or accidentally pollutes rivers, lakes, seas and coastlines [19]. Marine debris can be categorized into several diverse classes of material, including plastics, metal, glass, paper and so on [20]. Furthermore, plastic debris is a common component of garbage, and when not properly disposed of, it can end up in the environment, thus contributing to overall ocean pollution [21].

Degradation of plastics is difficult due to their xenobiotic origin and recalcitrant nature [22]. Global plastic production increased substantially to 311 million tonnes in 2014 and rose to 368 million tonnes in 2019 [23]. Plastics are generally inexpensive to manufacture and chemically resistant, which makes them difficult to degrade. They are used in various industries and products, including packaging, the food industry, medicine, electronics, textiles, and construction. Between 1950 and 2015, 6.3 billion tons of primary and secondary (recycled) plastic waste were generated. Approximately 9% were recycled, 12% incinerated, and the remaining 79% was either stored in landfills or released directly into the environment [24]. Approximately 40% of plastic packaging ends up in landfills, while 32% leaks from sewage systems [25].

A summary of the main categories of negative effects of plastics in the oceans allows for a basic understanding of the present problems facing marine life [26]:

1) Physical impacts: entanglement, suffocation, drowning, different injuries, and death through ingestion.

2) Chemical impacts: Plastic additives may release toxic products into the environment that can accumulate in the food chain.

3) Ecological impacts: ecosystem and habitat structure can be affected, and wildlife reproductive behaviour can be disrupted.

4) Economic impacts: industries, such as fisheries and tourism, can be heavily impacted through habitat destruction and loss of biodiversity.

Increased plastic pollution affects all levels of marine diversity [27]. They are widely distributed in northeast Pacific waters, including the "Great Pacific Garbage Patch," an area of debris captured by the North Pacific central gyre and well known for its high concentration of plastic debris [28]. Lost or abandoned fishing gear is also major plastic pollution in the oceans [29]. It accumulates in the environment and is broken down into smaller pieces through weathering. Nevertheless, efforts are being made to reduce the amount of plastic entering the marine environment [30] and thus reduce the danger they pose to marine life.

1.3 Microplastic pollution

Microplastics are small plastic particles less than 5 millimeters in size [31] that can be found in the form of fibres, beads, or shards, and are often used in products such as cosmetics, clothing, and packaging materials [32]. They can negatively impact a wide range of organisms, including fish, birds, and other wildlife, potentially entering the human food chain through the consumption of seafood and other sources, thus negatively that impacting human health [33]. The occurrence of microplastics is derived from the degradation of large plastic debris [34]. As of late, microplastics are a problem in oceans world-wide [35].

The presence and accumulation of microplastics in the ocean are of great concern partly because marine biota consumes them [36]. The sharp rise in the number of publications on microplastics in aquatic ecosystems observed lately justifies the great concern. Most research focused on quantifying microplastic abundance to understand these impacts better. Additionally, some researchers count the combinations of microplastic fragments that vary in size, shape, colour, specific gravity, chemical composition, and other characteristics.

1.3.1 The history of microplastic research

Microplastic research can be traced back to the 1960s when scientists first began to study the presence of small plastic particles in the ocean [37,38]. Research has revealed that microplastics have been found in various environments, including land, oceans, rivers, and even in the air. One of the major challenges in studying microplastics is the difficulty in detecting and measuring them [39-41]. Various methods have been developed to identify and quantify microplastics, including microscopy, spectroscopy, and chemical analysis [42-44].

Microplastic may threaten marine life, such as shellfish, fish, and cetaceans. They can be ingested directly from the surrounding water or by consuming prey that has ingested microplastics, thus considerably impacting the entire food chain [45,46]. Only a few studies have looked for microplastics directly in their digestive tracts [47]. The Indo-Pacific humpback dolphin (*Sousa Chinensis*) was the first cetacean species found with microplastics, showing that this pollution may threaten coastal ecosystems [48].

Many shellfish, such as Bivalvia, are very susceptible to microplastic contamination [49].

Research on blue mussels showed that they could ingest microplastics transported from the stomach to the digestive glands [50]. It is also reported that microplastics in the gills of blue mussels [50]. Table 1.1 summarises research confirming the presence of microplastics in shellfish. Most studies consider ingestion as the main route of microplastics into aquatic organisms showing that the digestive system has the major accumulation of these particles. Therefore, studying the microplastic distribution in the digestive system of shellfish is crucial because it can reflect its abundance in the environment [51].

Table 1.1 Studies dealing with microplastic contamination in shellfish worldwide [52].

Species	Countries	The lower limit of the filter pore size (µm)
Mytilus edulis	France	1.6
M. edulis* and *Perna viridis	China	1.6
M. edulis	Korea	20
P. viridis	India	11
Siliqua patula	United States	0.2
Theodoxus fluviatilis	Germany	0.2

One of the key areas of microplastic research has been the impact of these particles on human health. Some research reported that microplastics could enter the food chain from the ingestion by marine life, potentially negatively affecting other organisms' health [53,54]. The long-term effects of microplastic exposure in humans are not yet fully understood, but research is increasing in this area due to the increased public concern about the prevalence of microplastics in the environment. Governments and organizations around the world are starting to take action to address this issue, including banning or regulating their use in consumer products [55,56]. Overall, the research on microplastics has highlighted the need for a better understanding of their sources, impacts and effective reduction strategies.

1.3.2 Microplastics in oysters

Oysters are widely distributed in estuaries due to their high tolerance to salinity variation and pollution in general [57]. They are filter-feeding mollusks that settle gregariously and are directly exposed to contaminants in the water [58]. One oyster can filter 5-25 L of seawater per hour [59], and during this process, they are exposed to a high possibility of ingesting microplastics [60]. In brackish estuaries, most oysters are attached to hard surfaces such as rocks, mangrove roots, and other oyster shells [61]. They can also be found along the intertidal zone in many relatively shallow water, calm environments [62].

Oysters are famous worldwide as food. They also concentrate filtered particulate matter in excrement as bio deposits [63]. Oysters could capture and transport all sizes of microspheres and microfibres [64].

It is important to understand the presence and accumulation of microplastics in oysters and their possible implications for human health because they are consumed globally. Unfortunately, we still lack a strong enough data set related to the presence and consequences of microplastics in these organisms. The Pacific oysters (*Magallana gigas*) from the Southwestern Atlantic were reported to contain microplastics [65]. Moreover, oysters are suitable for studying microplastics and bioaccumulation because oysters are easy to culture in the laboratory [66]. Their widespread distribution, filter-feeding behaviour, and commercial importance, especially for aquaculture, make oysters ideal for studying microplastics in marine environments. For this thesis, quantities, and types of microplastics in oysters were recorded. They were assumed to indicate the level of potential microplastic distribution in the marine environment.

1.3.3 Microplastics in cetaceans

Cetaceans occupy the top trophic level in the marine ecosystem [67]. Cetaceans are catalyzed by Mysticeti and Odontoceti [68]. Mysticeti feeds by filtering large volumes of water through their baleen [69]. Odontoceti includes various species, such as all species of dolphins, all species of porpoises, and some species of whales like sperm whale *Physeter macrocephalus* and beaked whales [70]. One of the defining characteristics of odontocetes is their teeth, which they use to catch and consume their prey. Odontocetes are active hunters that feed on fish, squid, and other marine animals [71]. As apex predators, cetaceans are critical in regulating the food web and maintaining ecosystem balance.

Mysticeti has a filtering system in their intestines. This system is particularly important for species that feed on small prey, such as krill and plankton, which they consume in large quantities [72]. Intestinal villi trap and filter out unwanted particles or debris from their food [73]. Chemical functional groups such as carboxyl, amine, and hydroxyl in the intestine walls interact with and bind to various substances, including microplastics, thus removing them from the body [74]. Consequently, because the intestines are a key site for bioaccumulation, it is essential for microplastic research in cetaceans.

Bioaccumulation is the process by which a substance, such as a toxin or a pollutant, builds up in the tissues of an organism over time [75]. This occurs when an organism ingests a substance at a rate faster than it can metabolize or eliminate it, leading to the accumulation of the substance in the body [76]. One example of bioaccumulation is the case of polychlorinated biphenyls (PCBs), a group of synthetic chemicals commonly used in industrial applications [77]. The harmful effects of PCB bioaccumulation are the case of killer whales. Studies have shown that these whales have some of the highest levels of PCBs among marine mammals, and the chemicals have been linked to reproductive and immune system problems in the animals [78]. In addition, harmful substances, including heavy metals, can accumulate in animal tissues through bioaccumulation [79]. With the more harmful substances in the environment, including microplastics, animals such as cetaceans may accumulate more in their bodies. Cetaceans are more sensitive to microplastics.

At present, studies are not enough on microplastics in cetaceans. A study revealed the presence of an average of 5.5 particles per animal among the observed 50 stranded mammals [80]. A review [81] found that microplastics can interfere with the feeding and reproductive success of various marine organisms, including fish and invertebrates, which are food for cetaceans. Microplastics were reported in the gastrointestinal tract of bottlenose dolphins (*Tursiops truncatus*) stranded in South Carolina in a study covering materials from 2017 to 2018 [82] and were also found in the coastal species Indo-Pacific humpback dolphins (*Sousa chinensis*) [48]. As cetaceans rely on diverse food sources, microplastics in their prey may have cascading effects on cetacean populations. Therefore, further research on the prevalence and effects of microplastics in these animals is needed to help conservation efforts and shed light on the larger issue of plastic pollution in the oceans.

1.4 Impact of microplastics on seafood safety

A safe food supply is essential for a healthy society. The existing food systems are fraught with different types of risks. Food safety has been thought of narrowly, such as foodborne illnesses and risks directly related to food intake. A more comprehensive definition of food safety has been provided recently. Food safety refers to the measures taken to ensure that food is safe and free from harmful contaminants or substances that could cause illness or injury to consumers [83,84]. Zoonotic bacteria, viruses, parasites, and various physical hazards in food may pose safety risks to human health [85]. Among widely used food sources, seafood products are recognized globally for their high nutritional value and increasing popularity with consumers. Consumer preferences range from raw or minimally processed fresh products to prepared (salted, smoked, cured, canned) and ready-to-eat products. In addition, seafood is a major food category in international trade and is a major economic asset [86]. The importance of seafood safety will be discussed in the following section.

Seafood can contain many biological, chemical, and physical hazards. The most prevalent things are biogenic amines, biotoxins, and disease-causing bacteria and viruses [87]. More than 80% of seafood-borne outbreaks are associated with biotoxins (ciguatoxins), mackerel toxins, or the consumption of raw mollusks [88]. Some of the largest food poisoning outbreaks have been associated with seafood. Data on foodborne disease outbreaks collected over 10 years (1983–1993) in the United States demonstrated that fish were the third most reported category according to the vehicle of transmission [89].

On the other hand, there is concern about the possible impacts of microplastics on human health. Microplastic particles are ubiquitous in the environment, from the air we breathe to the food consumed by humans. Growing evidence suggests that humans are exposed to microplastics through food and drink consumption [90]. Chemical toxicity may be caused by organic and inorganic components of microplastics or by the exogenous harmful chemicals, proteins, and toxins they may carry [91-93]. A study mentioned in the question measured the exposure of European shellfish consumers to microplastics through dietary intake [94]. Therefore, studies of the potential threat of microplastics represented through the food web are needed to evaluate the possible food safety issues [95].

In conclusion, seafood is an important source of nutrition worldwide. It is essential to understand the potential risks associated with consuming seafood containing microplastics to protect public health. It is also important for consumers to be aware of these potential risks. Research on seafood safety and microplastics can help inform policies in order to develop regulations and strategies to reduce the number of microplastics entering the oceans, thus protecting seafood sources. Furthermore, cetaceans consume lower trophic-level organisms that have ingested microplastics. Therefore, studying the levels of microplastics in whales can provide an indication of the level of microplastic contamination in the larger marine food web, including seafood that humans consume. Consequently, research on seafood safety and microplastics are critical for protecting public health, the health of the oceans, and the sustainability of seafood as a resource.

1.5 Thesis objectives and outline

1.5.1 Main Objectives

The main purpose of this thesis was to study the presence and accumulation of microplastics in oysters and cetaceans, for better understanding their potential impacts on these species and the marine environment. In the case of oysters, the possible relationship between microplastics and food safety was discussed. The research involved analyzing the presence of fibres and their quantification in oysters and cetaceans from different locations and comparing the results to determine trends or patterns. The results of this research will contribute to the growing body of knowledge on the impacts of microplastics on marine life and the ecosystem. Obtained results can be used to inform efforts directed at the mitigation and management of this emerging environmental issue. Finally, the potential effects of microplastics on human health are summarised based on literature data analyzing the relations between plastic and toxicity. The conclusion discusses possible ways to reduce the impacts of microplastic pollution from the point of view of political measures and social awareness.

1.5.2 Thesis Outline

This thesis comprises 5 chapters.

Chapter 1 describes the current situation of marine pollution, especially microplastic.

Chapter 2 introduces a newly developed portable observation system used in this research.

Chapter 3 presents results from the analyses to detect and quantify microplastics in cultured oysters from the coastal areas of Japan.

Chapter 4 discusses the presence of microplastics in the intestines of stranded cetaceans.

Chapter 5 summarises the toxicity of microplastic from the viewpoint of quantitative analysis using published data.

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Chapter 2: Innovative Methodology

2.1 Observation methods for the study of microplastics

It is important to learn how to accurately identify and quantify the presence of microplastics in various samples. Here we describe an improved technique using the optical microscope and fluorescence microscope commonly used for microplastic observation techniques [1].

Optical and fluorescence microscopy enables researchers to identify and characterize the physical characteristics of microplastics, such as their size, shape, and composition. However, these techniques can be time-consuming and labor-intensive and may not be appropriate for large-scale studies [2]. Chemical analysis techniques, such as Fourier transform infrared spectroscopy (FTIR) and gas chromatography-mass spectrometry (GC-MS), are another method to detect the presence of microplastics [3] allowing the determination of the chemical compositions of microplastics [4]. The advantages and disadvantages of these methods are summarised in Table 2.1. However, not all observation techniques are appropriate for on-site observation. In other words, they are too heavy to carry to the field. Therefore, an innovative portable observation system was devised for this study.

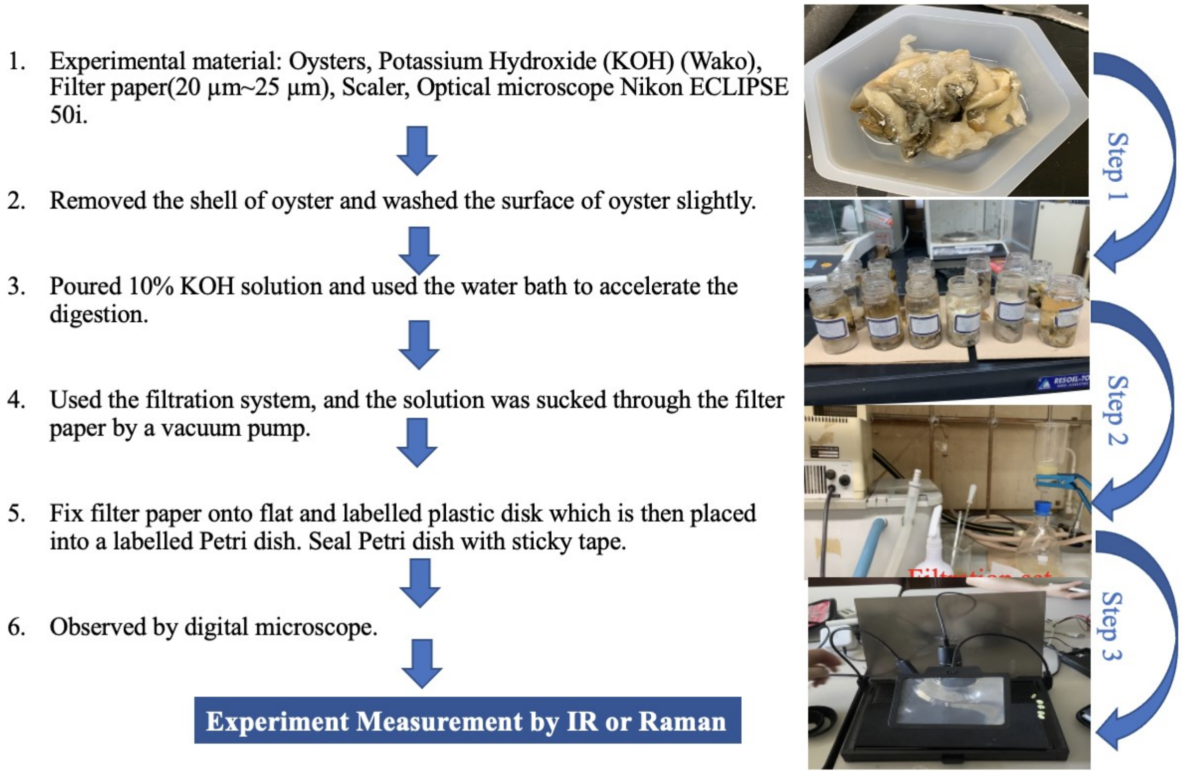
Table 2.1 Comparison of analytical devices [5-7].

Device name	**Advantages**	**Disadvantages**
Optical microscopy	Simple, fast, easy	No chemical confirmation – High possibility of false positive data – High possibility of missing small and transparent particles – No polymer composition data
FTIR spectroscopy	–Higher reliability concerning possible false positive data by chemical confirmation of all plastic-like particles – Reduction of false negative data –Nondestructive – Detection of less than 10 mm fibres –Automatic mapping (FPA reflectance)	– Expensive – Laborious work and time-consuming for whole particle identification –Contact analysis (ATR)
Raman spectroscopy	-Higher reliability concerning possible false positive data by chemical confirmation of all plastic-like particles – Reduction of false negative data – Detection of less than 1 mm of plastics – Nondestructive – Noncontact	–Expensive

2.2 Portable observation system

The new portable observation system used in this research consists of a sample stage, light sources, and digital microscope assembled with a Wi-Fi system. The specification of the digital microscope is 5.8 x 1.6 inches. The resolution is 1,920 x 1,080P (2 million pixels). There are eight adjustable LED lights on the end of the camera with excellent colour temperature in dark environments. Other features include portability, observation using various light sources, and simple handling. It is also very useful for fieldwork research. Wi-Fi enables the use of a smart phone or tablet instead of a computer.

Additionally, the sample stage has a drainage system for easy dissection of experimental samples. Another advantage is that it can be powered through USB mobile batteries. Consequently, it can function without an AC power supply. Figure 2.1 depicts the procedure of dissecting a sample (oysters are used as an example). The oyster shells were removed prior to the digestion experiment and the surface of the oysters was slightly washed. The tissue was then settled on the stage. After dissection, the gills and the digestive system were observed. Microplastics are easily visible under UV light. The surface of the intestine was also examined for microplastic detection using the same method. Following the digestion experiment, a digital microscope was used again to observe the microplastics on the surface of the filter paper.



1. Experimental material: Oysters, Potassium Hydroxide (KOH) (Wako), Filter paper(20 µm~25 µm), Scaler, Optical microscope Nikon ECLIPSE 50i.
2. Removed the shell of oyster and washed the surface of oyster slightly.
3. Poured 10% KOH solution and used the water bath to accelerate the digestion.
4. Used the filtration system, and the solution was sucked through the filter paper by a vacuum pump.
5. Fix filter paper onto flat and labelled plastic disk which is then placed into a labelled Petri dish. Seal Petri dish with sticky tape.
6. Observed by digital microscope.

Experiment Measurement by IR or Raman

Figure 2.1 Flow chart showing the use of the new portable system machines and chemical materials for the oyster study.

Fluorescence lights were used to observe the microplastics in the samples. In order to excite the plastics, a light source with a wavelength of 355 nm was used, and a 420 nm filter was mounted onto the lens of the digital microscope. During the observation process, it is necessary to switch filters with different wavelengths. As shown in Figure 2.2, several bright spots were observed, indicating the presence of microplastics in the sample. The optical magnification zoom can reach up to 1,000 times. The system can be used to observe microscopic objects invisible to the naked eye, monitor pores and skin, check cavities of animals and plants, close-up of electronic circuits, and machinery, and check substrates. The bottom right image shows the gills of oysters. Water and light reflections were found when the object was observed at different angles. Flexible arms are well suited to find good angles of observation. This procedure was applied in this research to observe oysters and cetacean intestine samples. This portable observation system allows the observation of both wet and dry samples which is very convenient. Furthermore, its numerous advantages allow for its use in the field.

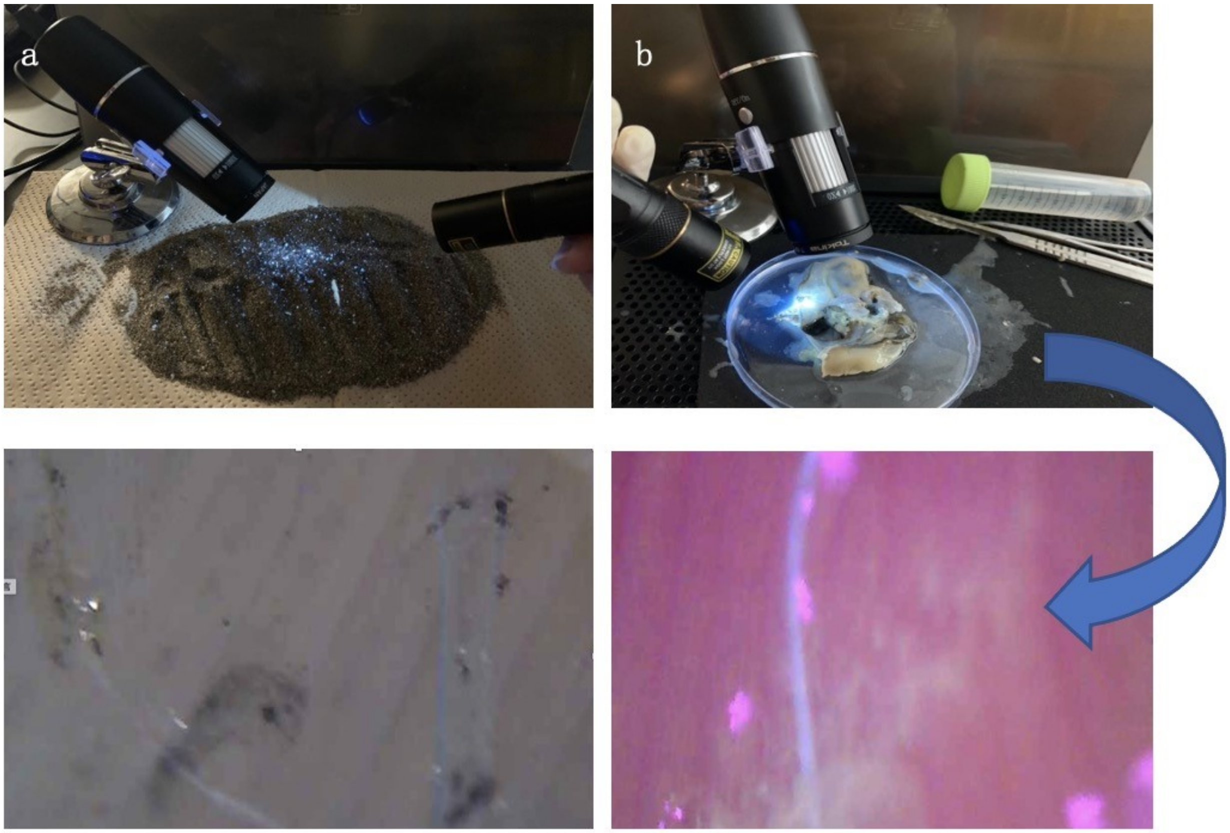


Figure 2.2 Typical microplastics found in oysters and sand: a) a digital microscope is used to observe the reflective parts in the sand under UV light. b) White and translucent blue fibres found in the gills of oysters.

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Chapter 3: Microplastics in cultured oysters from different coastal areas of Japan

3.1 Introduction

Studies dealing with the current extent of human impacts on marine ecosystems show that the oceans are becoming highly affected by human activities [1]. The accumulation of mismanaged plastic waste is now a global concern [2].

Since the 1970s, research on the presence of plastic debris in the ocean has shown an increase in the average concentration of plastic particles. For example, a study from the western Sargasso Sea indicated an average of 3,500 pieces and 290 grams per square kilometer [3]. Not being biodegradable in most cases, plastics widespread use constitutes a persistent environmental burden [4,5].

Plastic is a cheap, lightweight material that can easily be molded into various shapes. This has increased its annual world production from 1.7 million tons in the 1950s to 288 million tons in 2012 [6]. Our lives now seem to be inseparable from plastic. However, its negative effects, including many adverse effects on life and the environment, are becoming of the utmost concern [6].

Data show that most plastic waste in the oceans comes from terrestrial sources and is carried mostly by winds, wastewater treatment systems and road runoff [7,8], with the remainder coming from artifacts such as fish nets and ropes, aquaculture cages, and so on. [9]. Among microplastic garbage, plastic bags harm marine life the most [10]. Modern plastics are usually complex mixtures of polymers with chemical additives [11]. Plastic waste is usually colonized by bacteria [12] and often leaches chemical pollutants [13] into the waters. As mentioned, plastics are widely distributed on the surface and coastal waters of the global oceans, and their impact on marine life is enormous. For example, 44% of all seabird species are known to ingest plastic, as do sea turtles, cetaceans, and fish [14-16].

In recent years, more attention has been paid to microplastics [17], particles less than 5 mm that result from the breakdown of larger items [3]. Among the possible sources of these particles are waters from washing synthetic textiles discharged into the ocean. From the perspective of its composition, polypropylene (PP), polyethylene (PE), polystyrene (PS), polyvinyl chloride (PVC) and polyethylene terephthalate (PET) are the main components with higher probabilities of ending up in the ocean environment [18]. Indeed, several studies have found that both food and drinking water [19,20]. may contain different types of microplastics. They have also been found in human feces [21], increasing awareness of their possible effects on human health.

Compared with large plastics, microplastics are much more abundant and easily absorbed by organisms, thus increasing their toxicity potential. A Beijiang River surface sediment study found that microplastics were loaded with metals such as Ni, Cd, Pb, Cu, Zn, and Ti, mostly derived from inherent load [22]. Strong sorption of PCBs (polychlorinated biphenyls) to nano plastics and microplastics was also observed [23], as well as the presence of contaminants, such as polyfluoroalkyl substances (PFAS) [24].

Microplastics can also transport pollutants into marine food webs [25]. Moreover, in China, researchers established a model of microplastic accumulation in Baiyangdian Lake. The results showed that they spread and accumulate very quickly throughout the food web, eventually reaching high trophic-level aquatic organisms [21]. Studies from six locations along the French–Belgian–Dutch coastline looked at the absorption of microplastics under field conditions detecting them in mussels (*Mytilus edulis*) and lugworms (*Arenicola marina*). In France, microplastics were detected throughout the Bay of Brest, with polyethylene (PE, 53-67%), polypropylene (PP, 16-30%), and polystyrene (PS, 16-17%) fragments dominating both the surface water and the sediments [26]. Microplastic contamination of bivalves from the French Atlantic coast showed a high ratio of grey colour particles with sizes ranging from 50 to 100 µm in blue mussels (*Mytilus edulis*) and Pacific oysters (*Magallana gigas*) [27].

In Japan, microplastics were found in 31 out of 36 sites along 29 rivers where samples were collected in surface waters. Results show that the concentration of microplastics highly correlates with urbanization and population density and that the number of particles in rivers seems to depend on the human activities along the river basins [28]. Furthermore, microplastics were found in the digestive tract of 64 anchovies (*Engraulis japonicus*) in Tokyo Bay, representing 77% of the samples studied [17]. The correlation of microplastics' amount and human activities was also found in a study of coastal surface water done in the subtropical island of Okinawa, Japan [29].

Studies from the eastern coast of Australia seaports, report large amounts of microplastics in both sediments and oysters. Note that the density of particles in oysters was significantly higher than in sediments and black fibres between 0.1 mm–0.5 mm in size were the most abundant [30]. Experiments with oysters (*Saccostrea glomerata*) show that it can ingest and accumulate microplastics of 2 µm and 0.5 µm, that are then transferred through the membranes of the digestive glands to the haemolymph [31]. These studies emphasize the necessity to monitor microplastics in aquaculture and in the ocean, in order to enforce seafood safety.

Shellfish is an important component of marine ecosystems. Oysters are filter feeding organisms and as such they are especially easy to come into contact with microplastics [32]. Accumulating floating microparticles during the filtration process. Seafood products are important sources of proteins, polyunsaturated lipids, and phospholipids. Economic losses (Microplastic toxicity in marine organism, the cost of savage a huge amount of marine litter, etc.) due to microplastic pollution and marine debris in the marine environment are expected [33-35]. In Japan, oysters are very popular as food. With the development of the aquaculture industry, oysters (Mostly *Crassostrea spp*.) have become even more looked after. So far, Japanese researchers have predominantly studied viruses and their threat to oysters [36-38]. As the threat of microplastics to marine life becomes ever more serious, oyster aquaculture is expected to be highly affected.

Japan is an important oyster producer. Its oysters are exported worldwide and considered of prime quality. In this context, material from various aquaculture farms in Japan are studied in order to analyze the presence, quantity, size, and type of microplastics present to assess its the level in farmed oysters. Normally, farmed oysters are generally pre-washed, and UV irradiated before entering the market, so our research also aims to test the efficiency of these pretreatments on microplastics content. The possible sources of microplastics and its potential health risks are also discussed.

3.2 Materials and methods

3.2.1 Sampling sites

Oysters (*Magallana gigas*) were obtained at 12 locations along the Northern Island, Pacific Ocean, Sea of Japan, Inland Island, and Southern Islands. The objectives were to: (1) collect oysters from various agricultural farms and search for wild oysters near the coast; (2) take near-shore photos (environmental survey); (3) survey the surrounding living facilities (factories, living areas, etc.); and (4) compare the distribution of ocean currents. For this survey, the Fishery Adjustment Division of the Fishery Association Management Section of the Fisheries and Forest Department of the Hokkaido Government issued permission to take natural oysters. Fishery Cooperative Associations also helped to sample natural oysters in each area. The primary oyster breeding places in Japan were considered when choosing the sample sites for investigation.

The sampling also included cultured oysters bought from twelve sites (Table 3.1). This material was approximately two years old (personal communication from the seller). At least four oysters were sampled from each location. In order to avoid microplastic contamination, oysters were kept under freezing conditions, wrapped in aluminium foil for further experiments. A total of 106 collected samples were studied (Table 3.1).

During the collection of wild material, plastic waste was observed in a considerable amount of fishing nets and lines, as well as other plastic garbage abandoned on beaches near the areas where some of the studied material came. It is assumed that these discarded materials could be sources for the microplastics found in the studied materials.

Table 3.1 Location and average weights of oysters (without the shell) from different coastal areas of Japan.

	Location	Number of specimens	Average weight per oyster without shell (g)
S1	Northern island	10	30.50
S2	Pacific Ocean	20	35.19
S3	Northern island	5	41.50
S4	Pacific Ocean	5	24.39
S5	Pacific Ocean	5	19.95
S6	Pacific Ocean	4	49.68
S7	Sea of Japan	12	31.33
S8	Pacific Ocean	10	33.61
S9	Pacific Ocean	10	21.44
S10	Inland island	10	18.96
S11	Pacific Ocean	5	23.87
S12	Southern island	10	25.02

3.2.2 Methods

Microplastics were extracted from oysters following Chen's procedure [33]. The shells were removed, and the body's surface was slightly washed. The body was then placed into a 10% KOH solution, and a water bath was used to accelerate the digestion process. With the help of a vacuum pump, the solution was filtered through filter paper (20 μm-25 μm), and this was fixed onto a flat, labelled plastic disk and then placed into a labelled Petri dish. These were then sealed with sticky tape. A similar filtration procedure was applied to the dissected digestive tract of the oysters.

The dissected digestive and gill systems were observed through a digital microscope to assess the presence of microplastics (Figure 3.1) preliminarily. This simple procedure can be used to obtain images and evaluate the shapes and sizes of the microplastics. The colour can also be used to determine the presence of microplastics. In addition, tweezers were used to assess if the fibres would break. Raman spectra (RENISHAW inVia Raman Microscope) and optical microscopy (Nikon ECLIPSE 50i) were used to identify whether or not a particle was plastic. Raman methodology combines Raman spectroscopy and optical microscopy. It is one of the most efficient and effective ways to identify plastics by focusing a laser beam onto a small spot to obtain a spectrum. Raman spectroscopy can specify different polymers by probing different vibrational modes in the molecule.

Once a spectrum is acquired, the next step is identifying which polymers make up the unknown sample. A complete, high-quality spectral database is critical for accurate spectral matching. In our research, the software Open Specy was used. In this typical database, spectra of reference materials are recorded and stored with their chemical and physical properties in the sample record metadata. The spectra were matched with the spectra from the database. The software displays the highest matching polymer spectra. The spectra obtained through Raman were loaded into the gallery software, Open Specy, for analysis and identification of the plastic polymer type.

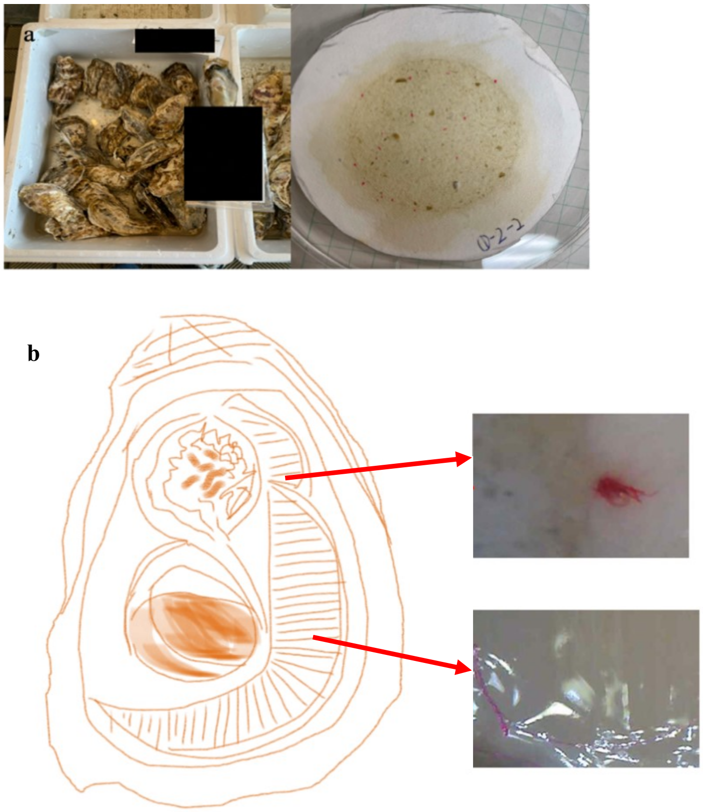
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Figure 3.1 a) Oysters purchased at seafood markets and local breeding bases. After the tissue digestion procedure, microplastics are shown in colour on the filter paper. b) Fibres can be observed in samples using digital microscopy before the tissue digestion procedure.

3.3 Results

Microplastics were found in almost all oysters studied. Our results indicate that microplastics were widely detected in cultured oysters in Japan. Microplastics (fibres and fragments mainly) were found in almost all oysters, and showed differences in length, size, and colour. A total of 333 microplastic pieces were collected from 106 oysters. The average number of microplastics per individual amounted to 3.14 ± 4.00 particles.

The average number of microplastics per oyster S4 material contains the highest value (up to 11.8 ± 8.5 items), followed by S5 (up to 7.8 ± 10.2 microplastics/oyster). The least microplastics per individual oyster come from S2, S3 and S10 (1.5 ± 1.5, 1.4 ± 1.3, and 1.5 ± 1.0, respectively) (Table 3.2).

The most common colours of typical fibres were red and green, whereas plastic fragments were mainly red and blue. Indeed, green particles constituted 57% of the total microplastics found, with red accounting for 17%. Fewer microplastics were blue (3%), grey or brown (About 5% in both cases). From the microscopic images, some fibre-formed clusters were observed (Figure 3.2, d). Sometimes, the edges of the fragment are damaged because of the degradation phenomenon (Figure 3.2, e).

Fibres were the most common type forming 88% of the total microplastics. The rest were mostly fragments. The most abundant size (32%) found was 100 to 500, followed by particles over 1000 (30%). Microplastics smaller than 100 were only 9% (Figure 3.3). The observation of dissected oyster gills and digestive systems confirmed the presence of microplastics in the gills of some oysters. The filtered material after the digestion of these organs also showed the presence of microplastic fibres (Figure 3.2). The analyses of the Raman spectra showed that the microplastics found in oyster samples are nylon 6, high-density polyethylene and polypropylene (Figure 3.4).

Pictures taken during the field work on several beaches show the presence of domestic garbage, as well as discarded fishing nets and lines (Figure 3.5).

Table 3.2 Microplastics in oysters from various locations in Japan.

Sample location	Number of specimens	Total number of microplastics per sample	Average number of microplastics per oyster
S1	10	24	2.4 ± 1.7
S2	20	30	1.5 ± 1.5
S3	5	7	1.4 ± 1.3
S4	5	59	11.8 ± 8.5
S5	5	39	7.8 ± 10.2
S6	4	30	7.5 ± 5.9
S7	12	33	2.8 ± 1.3
S8	10	29	2.9 ± 1.7
S9	10	19	1.9 ± 1.2
S10	10	15	1.5 ± 1.0
S11	5	12	2.4 ± 1.1
S12	10	36	3.6 ± 2.2
Total	106	333	3.14 ± 4.0

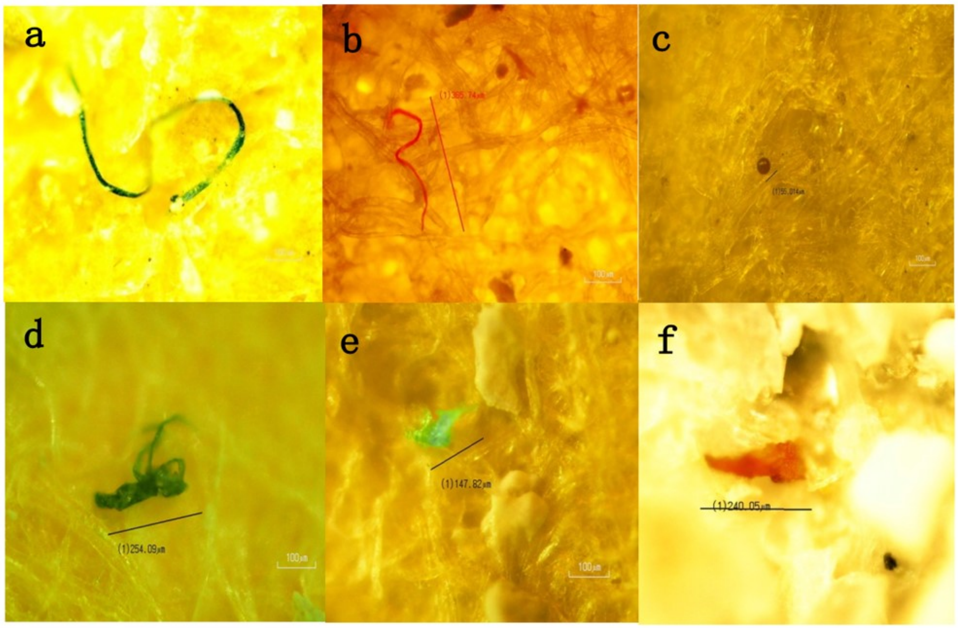

Figure 3.2 Typical microplastics found in oysters: a) green fibres; b) red fibres; c) brown bead; d) green fibres; e) green fragment; f) red fragment.

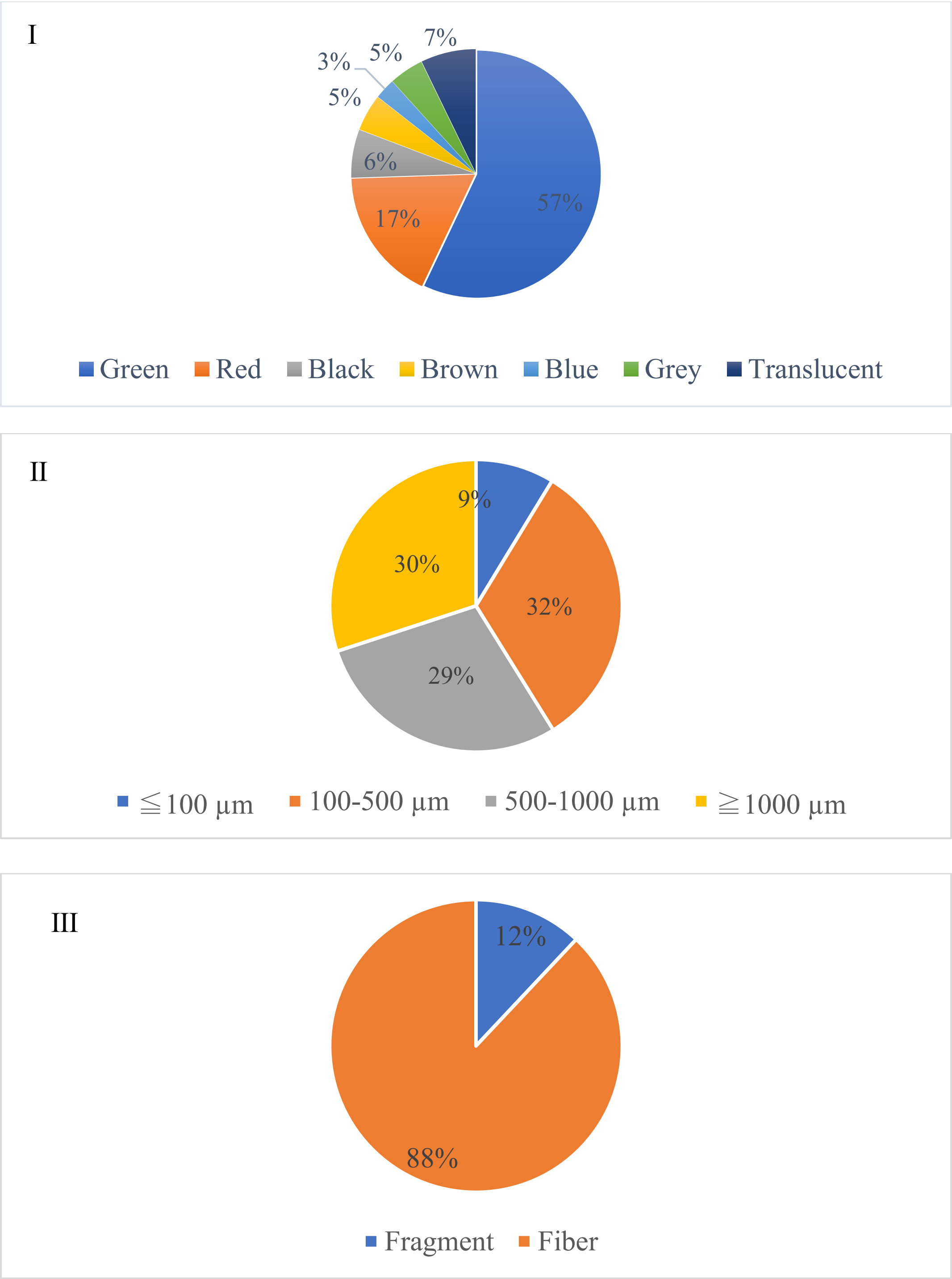

Figure 3.3 Distribution of microplastics found in oysters in Japan according to: (I) colour; (II) size; (III): shape.

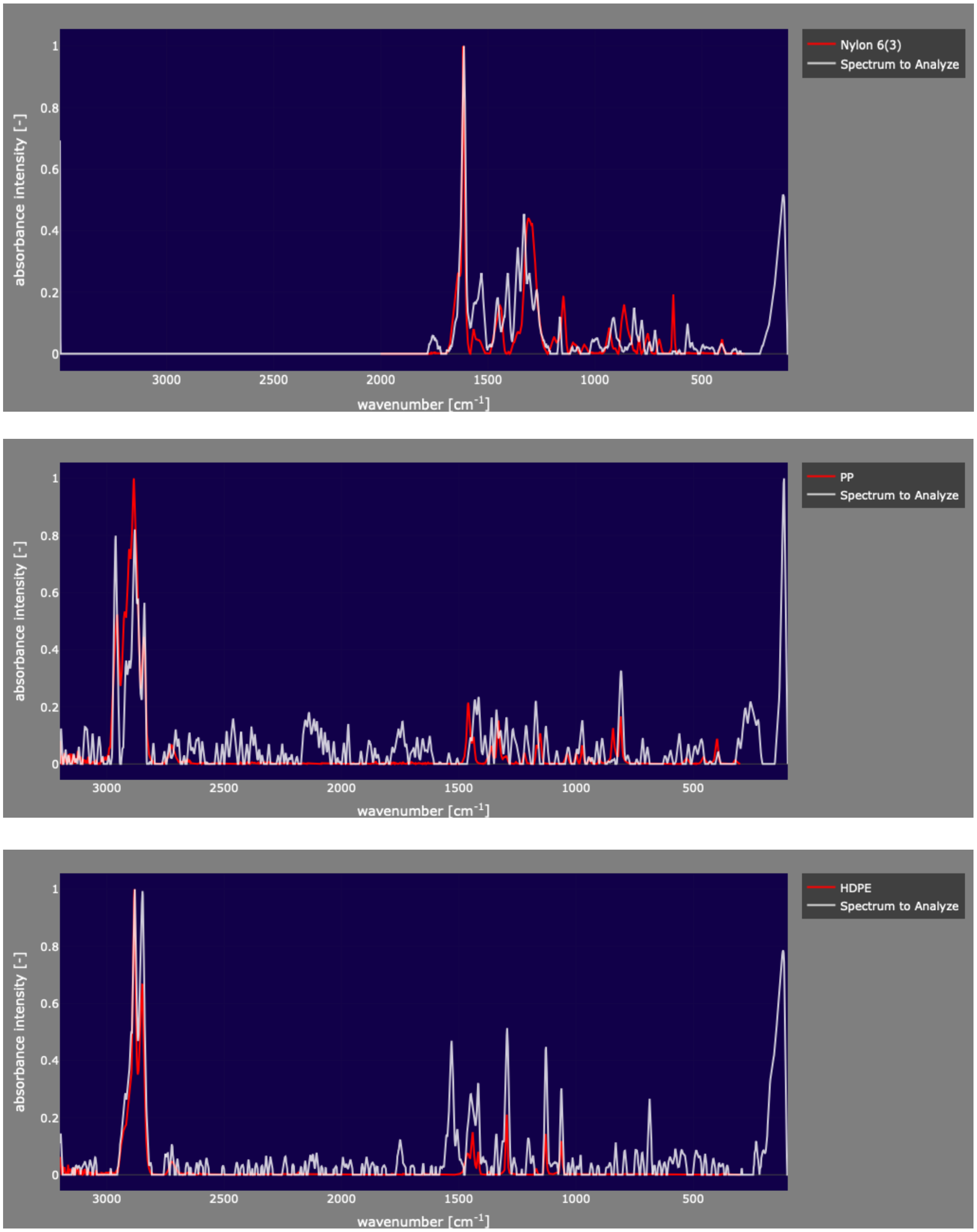

Figure 3.4 Raman spectra of selected microplastics: nylon 6, polypropylene (PP), high density polyethylene (HDPE).

Figure 3.5 Fishing gills, nets, and domestic garbage stranded on a beach. Hokkaido, Japan, August 2021.

3.4 Discussion and conclusions

The most common type of microplastics found in this study were fibres (88%) which agrees with data from 17 coastal sites in China where fibres constitute 60.67% of all microplastics found [22]. It should be noted that our material showed less diversity in the shape of plastics found. Microplastics found in this study can be divided into fragments and fibres (12% and 88%, respectively). Similarly, a study using oysters along the Pearl River Estuary in China indicated the dominance of fibres with a ratio of 69.4% and microplastic size in rock oysters (*Saccostrea cuccullata*) shows 83.9% of which were less than 100 µm. On the other hand, our research results show only 9% smaller than 100 µm, whereas the fraction from 100 to 500 µm has the highest proportion, up to 32%. In another study from the French Atlantic coast, the largest fraction of the particles found in the blue mussel (*Mytilus edulis)* and the Pacific oyster (*Magallana gigas*) ranged from 50 to 100 µm [39]. In Korea, four popular bivalve species, oyster (*Magallana gigas*), mussel (*Mytilus edulis*), Manila clam (*Tapes philippinarum*) and scallop (*Patinopecten yessoensis*) were evaluated, finding mainly fragments and particles smaller than 300 µm (76% and 65% respectively) of the total microplastics found [40]. In our study, fibres were the main shape of found.

In this study, the average of microplastics per oyster was 3.14 particles. This is less than what was found in *Perna viridis* (3.28 items/individual) in India (See Table 3.3 for a reference), but the most abundant type found was fibres, the same as in China. In both European and Asian literature, the size of microplastics found was smaller than that of Japanese oysters. On the other hand, the number of microplastics in Japan oysters was higher, and their size was larger than reported in other studies (Table 3.3). Regarding the type of microplastics identified using optical microscopy, most researchers use FT-IR and Raman spectroscopy, which gives confidence to our findings. Therefore, our data can be compared to that of other studies with high confidence.

From our observation, farmed oysters were cleaned and irradiated with ultraviolet rays before being sold. This procedure aims to reduce or eliminate possible microbial contamination of food. Although they were also cleaned using Milli-Q water to rinse both the shell and oysters' bodies before the digestion procedure, microplastics were found. All the cleaning methods done before the oysters are put on the market cannot remove the microplastics completely.

One of the problems concerns the source of the found microplastics. During the collection trips, a large amount of domestic garbage was found stranded on beaches (Figure 3.5). Note that the dates in some plastic packages were relatively recent. In addition, many fishing gills, nets, and cages used in aquaculture lay on the beaches. These are all made up of nylon. Table 3.2 shows that the average number of microplastics in S4, S5, and S6 is higher than in other sites. These sites are located on the eastern Pacific coast, so the pollutants' origin may be domestic garbage and that from neighbouring countries where ocean currents carry it.

Regarding S2, it is located in a relatively closed bay. This is also an aquaculture area, and local fishermen are more likely to pay attention to keeping the water-body healthy.

One research characterized microplastics in the heavily urbanized, brackish water of Vembanad Lake (India), focusing on some commercially important bottom-feeding fishes and shellfish (*Arius maculatus, Etroplus suratensis, E. maculatus and Villorita sp.*). Its results also showed the presence of polyethylene in the samples [41]. Polypropylene and nylon 6 were also reported in south India [42]. Similarly, the main polymers in shellfish from Dongshan Bay, southeastern China, were polyester and Polyethylene terephthalate [43]. The abundance and types of microplastics found exhibited great variation among species. Various microplastics are present in marine organisms due to their durability and wide range of applications in fishing activities and packing and textile industries.

Shellfish consumed as food may carry microplastics that will affect human health. As they are often consumed whole, the accumulation process in the consumers is a real possibility. Furthermore, plastics may have heavy metals and other toxic coatings that will carry on to the final consumer. These concerns lead us to recommend more studies of the possible pathologic effects of microplastics ingested with shellfish to understand their impacts in public health better.

Table 3.3 Studies showing microplastic contamination of marine life: location, type of digestion, identification method of microplastics, quantity, and abundant type with the corresponding references [22,44,45,46].

Species	Location	Digestion	Identification	Quantity	Most common types
Mytilus edulis	Germany	HNO_3 69%	Raman spectroscopy	0.36 ± 0.07 items/g (without depuration)	fibre and particle
Magallana gigas	France	HNO_3 69%	Raman spectroscopy	0.47±0.16 items/g (without depuration)	fibre and particle
Perna viridis	India	10% KOH	Fluorescence microscope	3.28 ± 0.87 items/individual	fragment
Meretrix meretrix	India	10% KOH	Raman spectroscopy	0.5 ± 0.11 items/individual	fragment
Mytilus galloprovincialis	Portugal	10% KOH	FT-IR spectroscopy	0.45 ± 0.67 items/individual	fibre
Scrobicularia plana	Portugal	10% KOH	FT-IR spectroscopy	0.30 ± 0.63 items/individual	fibre
Marphysa sanguinea	Portugal	10% KOH	FT-IR spectroscopy	0.40 ± 0.88 items/individual	fragment
Trachurus trachurus	Portugal	10% KOH	FT-IR spectroscopy	2.24 ± 2.05 items/individual	fibre
Scomber colias	Portugal	10% KOH	FT-IR spectroscopy	2.46 ± 4.12 items/individual	fibre
Magallana gigas *Crassostrea angulate* *Crassostrea hongkongensis* *Crassostrea sikamea*	China	10% (m/v) KOH + 30% H_2O_2	FT-IR spectroscopy	2.93 items/individual	fibre

3.5 Reference

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Chapter 4: Microplastic in the intestines of stranded cetaceans

4.1 Introduction

Cetaceans can be a soundness indicator of marine ecosystems because they occupy the higher trophic level of the ecosystem [1,2]. The cetacean study can provide current or potential impacts in marine ecosystems, helping to characterise strategies for dealing with the impacts [2,3].

Most cetacean species have ingested a wide range of plastics [4]. It ranges from microplastics, such as particles and fibres, to microplastics, such as plastic sheets, fishing nets, fishing lines, household items, etc. [see 5 for a review]. The International Whaling Commission considered plastic pollution an urgent issue since it may affect the mortality of cetaceans [6].

Two extant cetacean suborders have a large difference in the feeding ecology, affecting evaluating microplastics' effects in these organisms. Baleen whale (Mysticeti) species can be exposed to plastic pollution from direct uptake of the water and prey contamination as they filter a large amount of water while feeding [7-11]. On the other hand, toothed whales (Odontoceti), which hunt and swallow the prey whole or torn into pieces, should be more prone to be affected by plastics acquired through prey ingestion and trophic transfer [12-16].

Especially, ingestion of microplastics can occur directly from seawater filter feeding and indirectly from prey consumption [5,17]. It may lead bioaccumulation of microplastics [18-20]. A relatively high number of synthetic particles (mean value 0.057 particles/g) was found in krill ingested by fin whales on the western coast of Iceland [21]. Data from off the East coast of Asia found about 45 plastic particles in the body of a fin whale, including fishing lines, parts of fishing nets, plastic filaments, and Styrofoam pieces [22]. Several polymer types (polyethene and propylene, nylon, and polyethene terephthalate, among others) were all found in eastern north Pacific humpback whales (*Megaptera novaeangliae*) [17,23,24]. Blue whales (*Balaenoptera musculus*) in the Gulf of California feeding on krill are much more exposed to microplastic contamination through prey transfer than humpbacks which feed predominantly on fish [25].

Toothed (Odontoceti) whales are also commonly affected by microplastic pollution from the consumed prey. Beluga whales (*Delphinapterus leucas*) from the Canadian Northwest Territories were observed with a high amount of microplastic particles in their gastrointestinal tracts [26,27]. Similarly, a study of True's beaked whales (*Mesoplodon mirus*) stranded on the coasts of Ireland reported a large number of microplastics mixed with the remains of mesopelagic fish and cephalopods among their stomach contents [28].

It is crucial to increase our understanding of the ingestion of microplastics in cetaceans for various reasons. Firstly, cetaceans are apex predators in the ocean, and they can accumulate high levels of microplastics that can potentially damage their health and survival [29]. Furthermore, these species are valuable indicators of the soundness of marine ecosystems, so microplastics in their bodies can provide valuable insights into the extent and impact of marine plastic pollution [30]. Therefore, the description of microplastics in cetaceans is essential in understanding the impacts of microplastics.

Hokkaido is located in the Northern part of Japan, surrounded by the Pacific Ocean, the Okhotsk sea, and the Sea of Japan. The Northwestern Pacific Ocean is known for its high levels of microplastic pollution [31]. On average, 70 cetacean strandings are observed annually, and the Stranding Network Hokkaido collects and distributes the specimen of stranded cetaceans for academic purposes.

The objective of the study in this chapter was to evaluate the presence of microplastics in cetaceans collected in Hokkaido, Japan, in order to increase the available information related to their potential impacts on marine ecosystems.

4.2 Materials and methods

4.2.1 Material

Specimens collected by the Stranding Network Hokkaido (SNH) were used for this research. SNH is a non-profit organisation aiming to collect information and specimens of cetacean strandings, including beaching, drifting, and bycatch, established in 2007. They called on the general public, administrative bodies, fishery officials, and others to report information on stranding to the dedicated receiving and reporting desk 'IRUKA KUJIRA 110' and collected an average of about 70 cases per year, with a cumulative total of over 900 cases of stranding information by 2021 [32]. Upon receiving information on stranding, SNH investigates the approachability and condition of the specimen and determines the feasibility of conducting a survey. If a survey is deemed feasible, permission is obtained from the relevant authorities, and the survey is carried out. The survey may involve a visit to the site for a full body recovery, or the survey may be carried out on-site where the specimen has drifted ashore or at the disposal site. In either case, specimens are collected by searching for external morphological information and external injuries. The SNH obtains requests in advance from the research institutions for the samples needed for the study. When the specimens are available, SNH sends specimens to the institutes free of charge and unconditionally [33]. The use of stranded specimens allows these studies to proceed without unnecessary collections of living specimens, thus contributing to conservation efforts.

Intestines of 11 cetacean specimens were obtained from different locations in Hokkaido. Table 4.1 shows the detailed information for each cetacean specimen used in this study. A total of 17 samples were prepared and analysed for microplastic detection. A five-centimetre sample was taken for each received intestine, keeping in mind that the sample was clean from sand and sediment. In 3 cases, plural samples were obtained: SNH21007 (five samples), SNH20091 and SNH190032 (two samples each). In order to avoid contamination, samples were wrapped in aluminium foil and frozen before the analysis. The study of carcasses can be complicated due to uncertainties in determining the stage of decomposition. However, it is crucial to have a system in place to define the quality of the material being studied. The code system was established by the Smithsonian Institution's Scientific Event Alert Network. In Table 4.1, CODE 2 points to carcass in good condition. CODE 3 means the carcass was decomposed, but organs basically intact [52]. In the research, the experimental intestines were proved to be eligible.

Table 4.1 Stranded locations and species of sampled cetaceans.

Sample ID	Stranded Location	Species Name	Common Name	Suborder	Body length	Sex	The stage of decomposition
SNH20091	41.986064N 140.906982E	*Balaenoptera acutorostrata*	Minke whale	Mysticeti	495.0 cm	Male	CODE 2
SNH19001	41.868298N 140.115493E	*Lagenorhynchus obliquidens*	Pacific white-sided dolphin	Odontoceti	219.9 cm	Male	CODE 3
SNH20032	41.940520N 143.242260E	*Stenella coeruleoalba*	Striped dolphin	Odontoceti	176.0 cm	Male	CODE 2
SNH21060	42.389176N 141.084797E	*Stenella coeruleoalba*	Striped dolphin	Odontoceti	191.1 cm	Female	CODE 2
SNH21052	42.600722N 141.488556E	*Stenella coeruleoalba*	Striped dolphin	Odontoceti	196.9 cm	Female	CODE 2
SNH22001	42.010660N 140.104330E	*Lagenorhynchus obliquidens*	Pacific white-sided dolphin	Odontoceti	202.4 cm	Male	CODE 3
SNH22006	41.468139N 140.029861E	*Phocoenoides dalli*	Dall's porpoise	Odontoceti	196.5 cm	Male	CODE 3
SNH21066	42.619847N 141.564035E	*Stenella coeruleoalba*	Striped dolphin	Odontoceti	215.7 cm	Male	CODE 2
SNH21007	42.697500N 140.051944E	*Phocoena phocoena*	Harbour porpoise	Odontoceti	178.6 cm	Female	CODE 3
SNH22016	42.331027N 141.022174E	*Phocoena phocoena*	Harbour porpoise	Odontoceti	136.1 cm	Female	CODE 2
SNH21036	42.393920N 140.906860E	*Stenella coeruleoalba*	Striped dolphin	Odontoceti	186.8 cm	Female	CODE 2

In order to ascertain if all plastics emit fluorescent light and can thus be observed under a fluorescence microscope, a test experiment was done. Small pieces (less than 5 mm) were cut from several plastic items commonly used in household and industrial items, marked and placed in one of the intestine samples. This sample was then examined under a fluorescence microscope using the same conditions as the intestine samples used in this study. The materials used for this test were: Polyurethane (PU), Polyethylene terephthalate (PET), Poly (methyl methacrylate), i.e. acrylic plastic and plexiglass (PMMA), and Polystyrene, i.e. foams (PS). Observed microplastic fibbers were characterised through their size, colour, shapes and possible origins. Furthermore, the chemical composition (polymer types) of the found fibres was identified using Raman analysis.

4.2.2 Methods

Raman spectroscopy is a useful tool for the analysis of microplastics. It can provide specific information about the chemical composition and structure of a sample without the need for sample preparation or destruction. This makes it a non-destructive and efficient method for identifying microplastics [34]. Another merit of Raman spectroscopy is its ability to differentiate between different types of polymers based on their unique Raman spectral signatures [35]. Based on their characteristic peaks, the spectra of polyethylene, polypropylene, and polystyrene can be easily separated. Raman spectra (RENISHAW inVia Raman Microscope) were used to identify the microplastics found. The measured spectra were uploaded to Open Specy and compared with those in the database library. Open Specy automatically displays the highest matching polymer spectra, thus allowing the identification of the studied sample.

In order to find microplastics more efficiently in the studied samples, the observation methods were divided into three types.

a) Digestion

Microplastics were extracted from the intestines following Chen's procedure [36]. The surface of the intestines was first lightly washed with Mili-Q water. They were then placed into a 10% KOH solution, and a water bath was used to accelerate the digestion process. With the help of a vacuum pump, the solution was filtered through filter paper (20–25 µm), which was then fixed onto a flat. Labelled plastic disks were placed into a labelled Petri dish and sealed with sticky tape. An optical microscope (Nikon ECLIPSE 50i) was used to preliminary identify the particles to assess if they were plastic. Observations were made under 100x magnification. Obtained images were used to evaluate the shapes and sizes of the observed microplastics.

b) Direct observation

After the intestines were washed with Mili-Q ultrapure water, they were opened and flattened with the help of clips to expose the inner wall (Figure 4.1). A digital microscope was used to search for larger plastic fragments in the sample under a magnification of 200x**.**

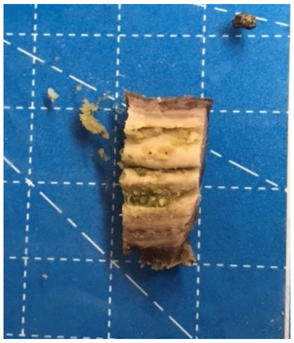


Figure 4.1 Dissection of the intestine to expose the inner wall.

c) Fluorescence microscope

Fluorescence microscopy was used to reduce the likelihood of missing microplastics in the studied samples. A cryostat (Leica CM 3050S) was used to slice the samples for further observation under a fluorescence microscope. Figure 4.2 shows the stages of this process.

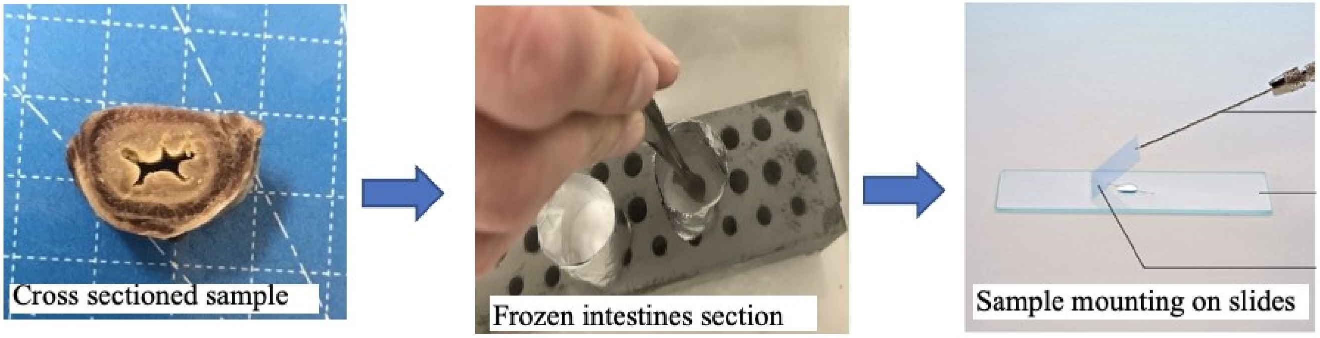


Figure 4.2 Flow chart of the intestines' sample preparation for observation under the fluorescence microscope.

4.3 Results

Some microplastics were found in 13 out of 17 samples of cetacean intestines (Table 4.2). The two samples without microplastics were from SNH20032 and SNH22006 (Table 4.2). Observed microplastics showed differences in length, size, and colour (Figure 4.3). Most microplastics observed were fibres (Figure 4.4). Only Nylon 6,6 was identified by Raman spectroscopy (Figure 4.5). Figure 4.6 shows the test experiment results using common household items made of different plastic polymers.

Table 4.2 Microplastics presence in stranded cetaceans from various locations in Japan. For species names and detailed location information, refer to Table 4.1.

Sample ID	Number of samples taken	Observation of microplastics in the samples
SNH20091	2	Presence
SNH19001	1	Presence
SNH20032	2	Absence
SNH21060	1	Presence
SNH21052	1	Presence
SNH22001	1	Presence
SNH22006	1	Absence
SNH21066	1	Presence
SNH21007	5	Presence
SNH22016	1	Presence
SNH21036	1	Absence

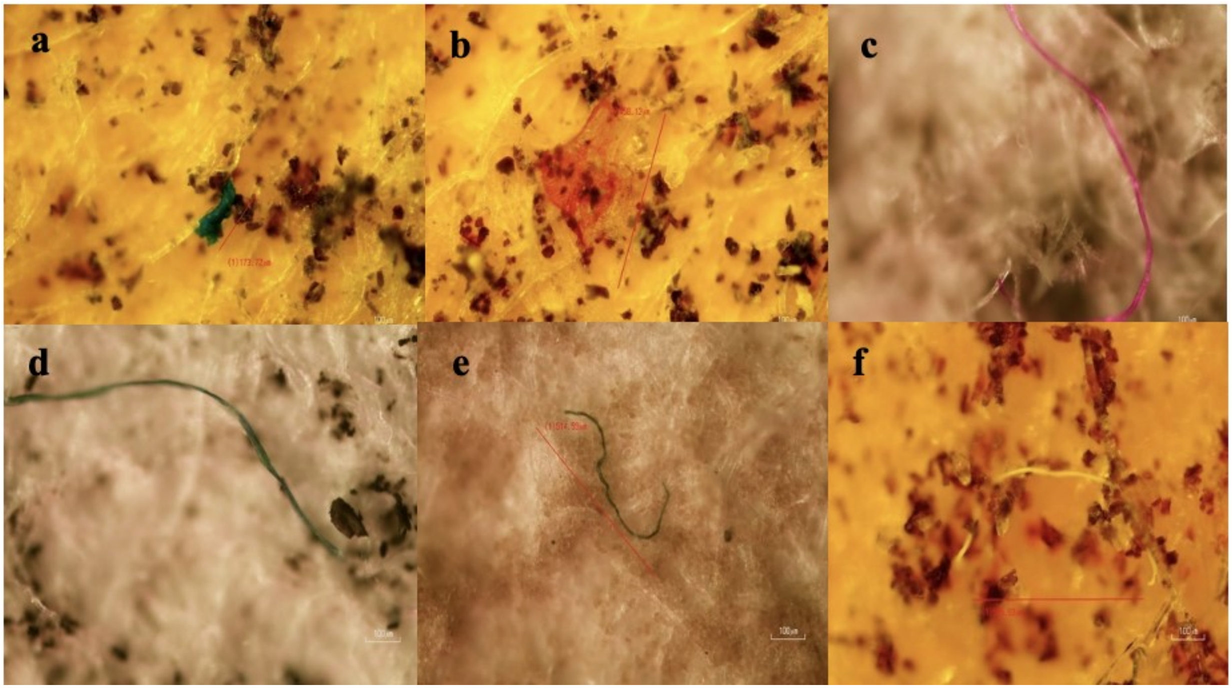


Figure 4.3 Typical microplastics found in cetacean intestines: a) green fragment; b) red fragment; c) pink fibre; d) green fibre; e) green fibre; f) yellow fibre.

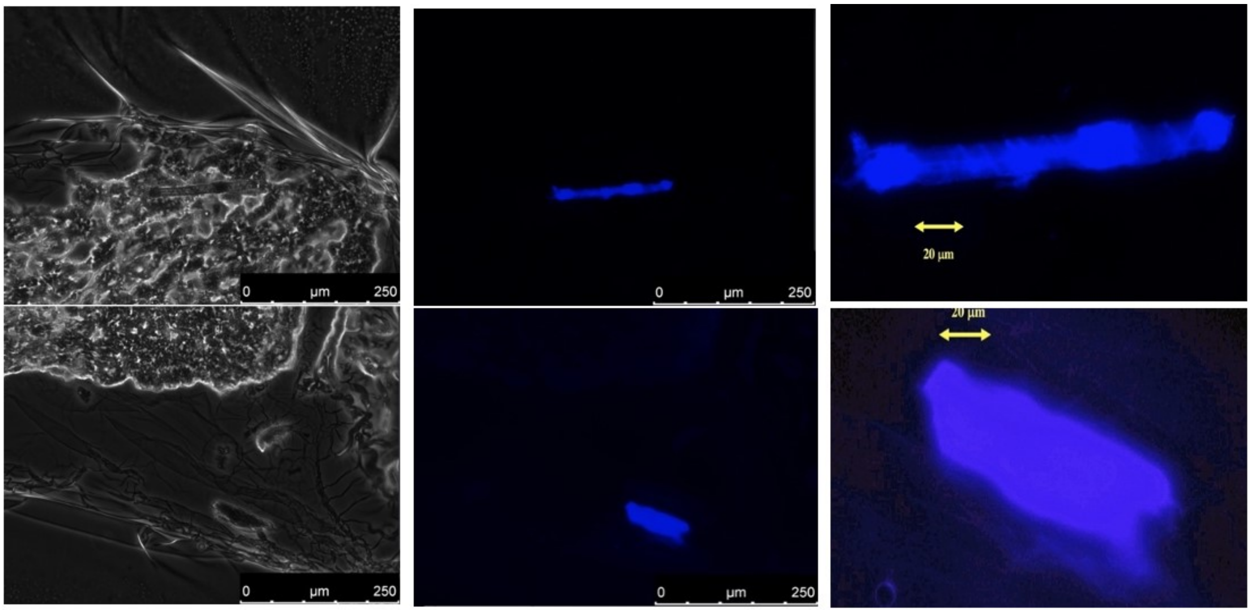
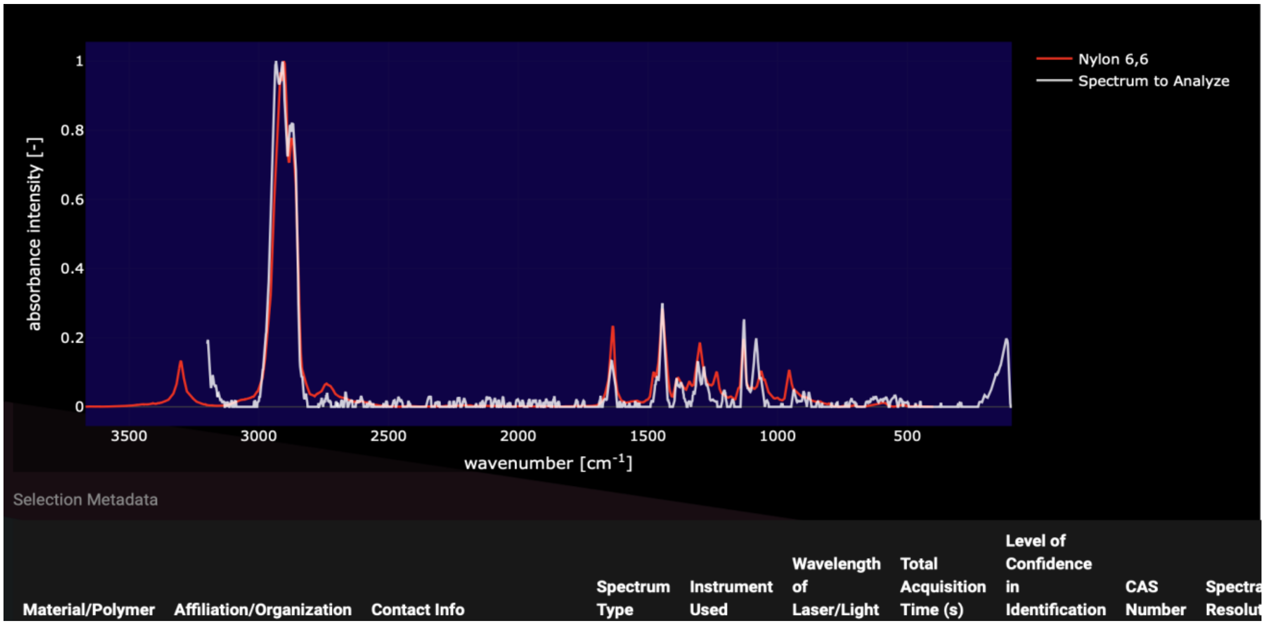


Figure 4.4 Detail images of fragments and fibres from cetacean intestines taken with a fluorescence microscope.



Material/Polymer	Affiliation/Organization	Contact Info	Spectrum Type	Instrument Used	Wavelength of Laser/Light	Total Acquisition Time (s)	Level of Confidence in Identification	CAS Number	Spectra Resolut

Figure 4.5 Raman spectra of Nylon 6,6.

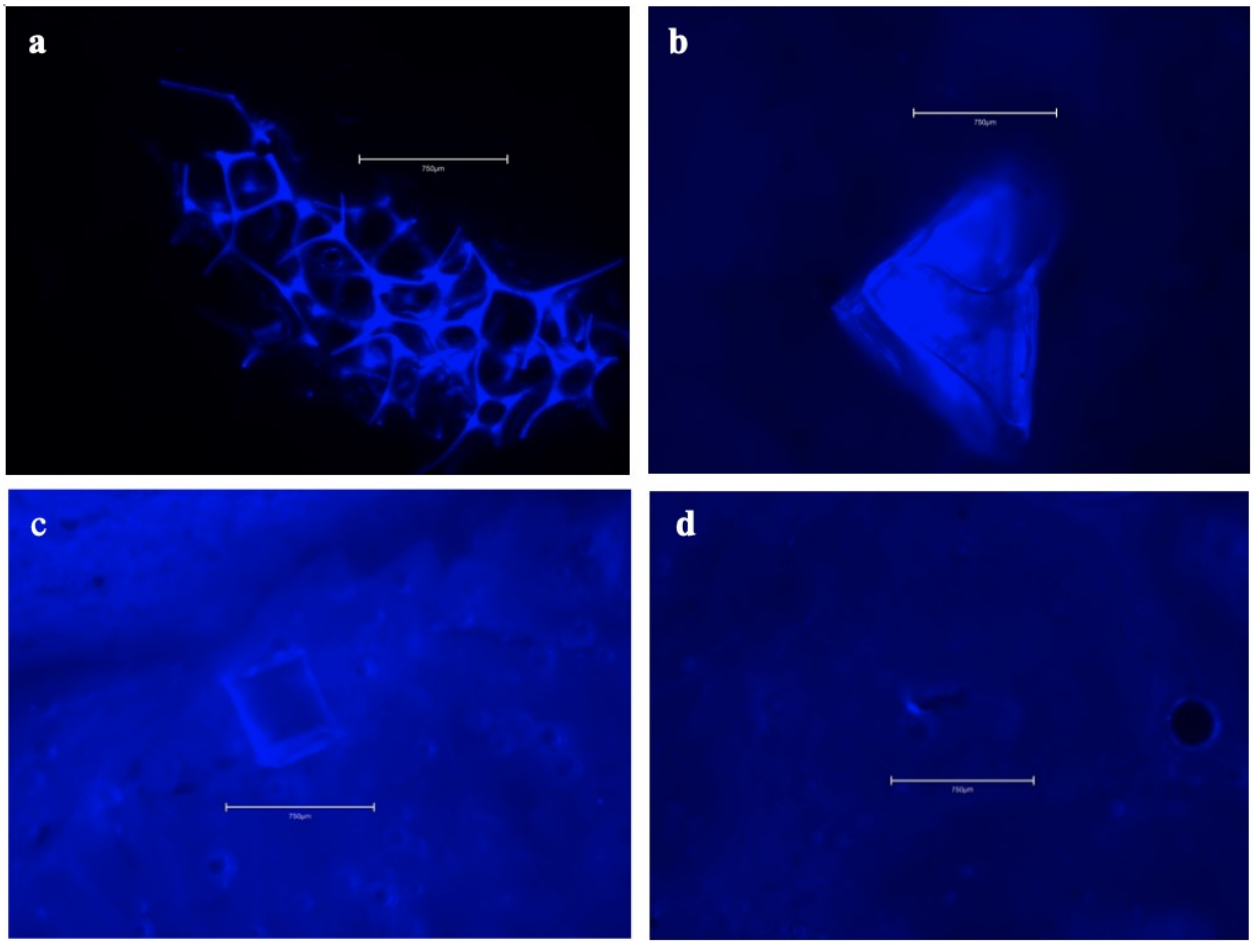


Figure 4.6 Images of the test done with different materials. a) PU (Polyurethane) and b) PET (Polyethylene terephthalate) are very obvious under the fluorescence microscope. c) PS (Polystyrene) and d) PMMA (Poly (methyl methacrylate)) cannot be distinguished from the surrounding tissue under the fluorescence microscope.

4.4 Discussion

Our study aimed to ascertain the presence of microplastics in the intestines of stranded cetaceans in several areas of Japan and their characterisation. Plastic production and its use are continuously increasing [37] despite the alarming data from many studies [38]. Indeed, plastic pollution and its consequences for marine diversity, in general, have been widely documented [6,17, 39]. Although observations of microplastic ingestion in cetaceans have increased [see 24 for a review], they are still lagging behind those of other marine groups. Cetaceans are top predators and can be well positioned as sentinel species to evaluate the health of the world's oceans [1-3]. Thus, it is crucial to increase our knowledge of the consequences of plastic pollution to develop better recommendations and guidelines for this important group of marine organisms.

As mentioned before, cetaceans' feeding habits range from top carnivore species (belonging to the toothed or Odontoceti suborder) through filter-feeding baleen species (Mysticeti suborder) [6,17]. Microplastic particles were found in most analysed samples through fluorescence and optical microscopy. The small amount found may be related to the fact that the studied material consisted mostly of toothed cetacean species. Indeed, only one baleen species was available for this study. Toothed whales are more exposed to macroplastics because of their feeding ecology [26,40]. Indeed, predictions based on observations and modelling consider that fish and cephalopod-feeding toothed cetaceans would be less impacted by microplastics when compared with krill-feeding baleen species [25]. The highest number of observed microplastics were fibres which agrees with several other studies in cetaceans and other marine vertebrates [41,28,42].

In order to compare data, the same methodology should be followed if at all possible. Unfortunately, we could not access the instruments usually used for this type of study. Consequently, it was decided to use fluorescence microscopy. The use of this method to observe microplastics in cetacean intestine tissue with cetaceans has not been reported in previous studies, so this also had value as a methodological first trial. Even though fluorescence microscopy could clearly capture microplastic images, sample processing was long and inefficient. Therefore, only one sample was used for this trial, confirming that fluorescence microscopy can be used to obtain clear images. We would recommend this method for studies that work with smaller samples.

The small number of microplastics found could be due to several reasons. First, fluorescence microscopy may not be the most efficient method to observe microplastics in the intestines of marine mammals. Fluorescence microscopy is normally used to detect microplastics in water [43-45]. However, many microplastics have low intrinsic fluorescence signals or lack them together [46-48], making their observation quite challenging. Figure 4.6 shows that while Polyurethane and Polyethylene terephthalate has strong fluorescence emission signals, the signals are very weak and cannot be observed with fluorescence microscopes. A way to improve the fluorescence emission signals is to stain the samples before observation using fluorescent dyes such as Nile Red [49]. We wanted to check the reliability of this method in tissues. Unfortunately, our samples were very brittle after being processed for the microtome, so they could not be used further to separate the microplastics from the medium. Therefore, we relied on the digestion method to assess the presence of microplastics in the samples.

It is generally difficult to compare studies of microplastic contents in cetaceans due to methodological issues. As explained above, the use of fluorescence microscopy was chosen due to the lack of other instruments usually referenced in other reports. Unfortunately, our experiments with this method did not yield reliable results, and less-used methods were used, such as the digestion of tissues. Furthermore, some studies examine both stomach and intestines [28,42], while others use subsamples to extrapolate the total potential amount [27]. This study only used intestines which maybe was not enough to detect microplastics. Several studies report blue and black as the most frequent microplastic colours [41]. On the contrary, this study found mostly green fibres and fragments (Figure 4.3). We also found red and yellow fragments.

This study was the first observation of Nylon 6,6 in cetaceans [50]. This robust plastic, often used for pelagic fishing nets, is considered a common source of ocean microplastics [41]. It should be observed that Nylon is a very common polymer found in ocean sediments as well as macroalgae beds [51]. More studies are necessary to assess this polymer's possible transference to the animal food chain in the oceans.

As plastic pollution increases in the oceans, we need more data from ecologically critical prey species to accurately characterise microplastics' danger. New data using different species at all trophic levels would allow a complete understanding of plastic presence in marine food webs and its consequences for apex predators, including predatory fish, seabirds, and marine mammals.

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Chapter 5: General conclusion

The study detected microplastics in cultured oysters from Japan, mainly in the form of fibres and fragments with red and green being the most common colours. Fibres were the most common type, with an average of 3.14 ± 4.00 particles per individual. Microplastics were found in oyster gills and digestive systems, and the Raman spectra analysis showed they were nylon 6, high-density polyethylene and polypropylene. Furthermore, Microplastics were detected in 13 out of 17 samples of cetacean intestines. The observed microplastics showed variations in length, size, and colour, most of which were fibres. Raman spectroscopy analysis identified Nylon 6,6 as the sole polymer present in the microplastics.

The thesis investigated the presence and accumulation of microplastics in oysters and cetaceans. The potential impacts on these species and the marine environment were discussed. The research also examined the potential relationship between microplastics and seafood safety in oysters. Additionally, the thesis analyzed the potential effects of microplastics on human health based on existing literature and discussed possible measures to reduce the impact of microplastic pollution from a political and societal perspective. The results of the study will contribute to the current knowledge of the impact of microplastics on marine life and ecosystems and can aid in developing strategies to mitigate and manage this environmental issue.

5.1 Toxicity of microplastic

When researchers discuss the social impact of microplastics, different aspects of their situation in life need to be considered. Studies of microplastics in various organisms were summarised. Micro pieces have been proven to be ubiquitous in the environment [1,2]. In recent years, microplastics have accumulated in oceans and sediments worldwide, reaching a maximum capacity of 100,000 particles per m^3 [3]. Because of their small size, microplastics can even be ingested by microorganisms [4]. However, the food chain is a more important means of transporting and storing nutrients. Plastic fragments, spheres, and fibres are already consumed by commercially available species such as marine molluscs, crustaceans, and fish. A growing body of research indicates that microplastic ingestion directly or indirectly affects marine organisms [5,6]. Researchers have found microplastics in the intestines of four kinds of seafood: cod, dab, flounder, and mackerel. These four kinds of fish are seafood with a high sales rate in the market [7]. The social impact of microplastics is also very crucial.

Regarding pathology, the toxicity of microplastics will cause oxidative stress, including disturbance of redox balance and damage to cellular components (Apoptosis) [8]. Because of microplastic, the proliferation of small vessels and infiltration of chronic inflammatory cells happened (Inflammation) [9]. Microplastics directly interfere with the normal synthesis of ATP. This phenomenon directly induces mitochondrial depolarization. Microplastics can interfere with the normal metabolism of ATP (Adenosine triphosphate) [10]. Nanoparticles (microplastics) of different sizes entered into cells. Confocal microscopy revealed that the entry route of these particles changed from direct penetration to endocytosis with increasing particle size, and then subcellular localization changed as well. The researchers hypothesized that DNA damage and repair balance were broken [11] (Figure 5.1).

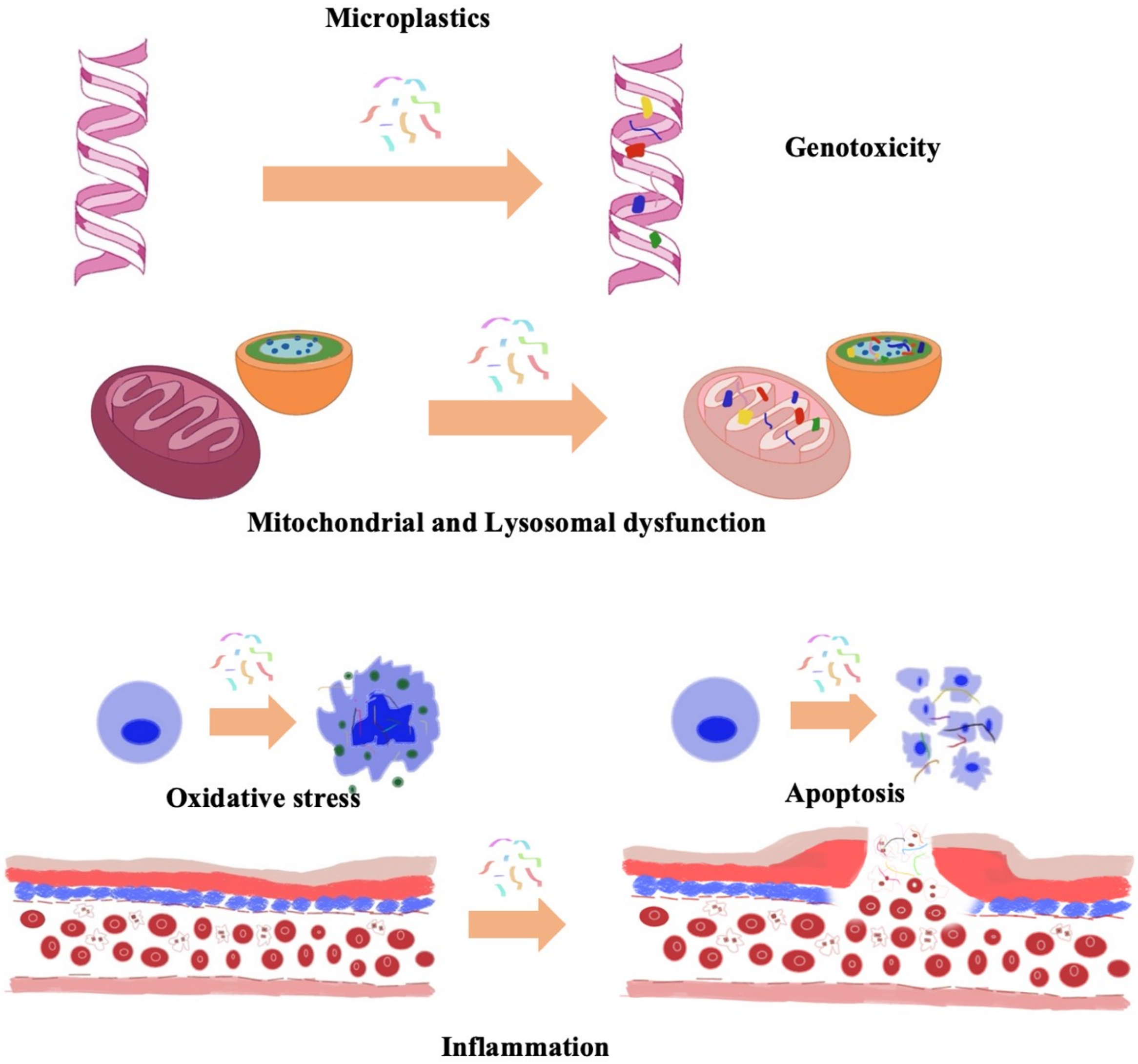

Figure 5.1 Polystyrene microparticles show oxidative stress, apoptosis, inflammation, mitochondrial and lysosomal dysfunction, and genotoxicity in cell cultures [12].

Studies have shown that microplastics may cause inflammation, mitochondrial dysfunction, lysosomal dysfunction, and apoptosis [12]. Toxic effects depend on the dose used in the experiment, the dose rate, and the duration of exposure [13-15]. For humans, microplastics are everywhere. Studies have shown that there are also many microplastics in the air [16-18]. A study summarises data on microplastics found indoors. They also calculated the estimated daily intake of MPs from inhaling indoor dust. The adult consumes 0.23 MPs/kg BW/day [19]. The reference shows that microplastics are estimated to account for 33% of the fibres in indoor environments [20]. In the outdoors, one study found six different microplastics in Australian road dust: polypropylene, polystyrene, polyethene terephthalate, polyvinyl chloride, poly (methyl methacrylate), and PE. Their densities range from 0.5 to 6 mg/g [21]. More microplastics are in the air than humans ingest accidentally by eating an oyster (3.14 particles per oyster). It can be inferred that microplastics in oysters do not pose a significant threat to human health.

It is difficult to determine a specific threshold at which microplastics begin to pose a threat to oyster mortality. According to the data on oysters in the current study, the average microplastic of an individual oyster is 3.14. The microplastics observed are nylon 6, nylon 6,6, polypropylene (PP), and high-density polyethene (HDPE). The density of nylon 6,6 is 1.14 g/cm^3 [22], nylon 6 is 1.08 g/cm^3 [23], and polypropylene is 0.946 g/cm^3 [24]. HDPE is well known for its high strength-to-density ratio. The density of HDPE ranges from 930 to 970 kg/m^3 [25]. The average length of all microplastics was 593.23 µm. The average diameter of the microplastics is 10 µm. According to the data, 88% of microplastics are fibres. The average volume of microplastic can be obtained through cylindrical calculation. The average volume of the microplastic is 46,568.56 µm^3 (4.66×10^{-8} cm^3). In an old study, researchers injected nylon 6 into mice. When the injection dose was 500 mg/kg, convulsions were observed in mice. Mice died at injection doses of 800 mg/kg (per body weight of mice) [26]. Another experiment revealed that when rats were given oral administration of over 5,000 mg/kg of HDPE, toxicity occurred, and rats died [27]. Nylon-66 has been approved as indirect food additives as polymers used for food contact surfaces [26]. Fiume et al. previously reported that adipic acid (5%) was not carcinogenic in rats fed diets [28,29]. Polypropylene is a colourless, odourless granular solid. The lethal dose for rats was determined to be 5,000 mg/kg [30]. The average weight of oysters is 29.62 g. An oyster will die if it ingests at least 0.02 g of nylon 6. The oyster would die if it consumed over 0.15 g of PP or HDPE. In table 5.1, the pollution level of microplastics observed in the oyster was much lower than the lethal level.

Table 5.1 Comparison of the lethal dose of plastics and the weight of microplastics in oysters.

Name of the microplastic	**Density**	**Weight of microplastic**	**Lethal dose**
Nylon 6	1.08 g/cm^3	1.59 × 10−7 g	0.80 g/kg
Nylon 6,6	1.14 g/cm^3	1.67 × 10−7 g	N. A
Polyethylene	0.95 g/cm^3	4.37 × 10−8 g	5. 00 g/kg
Polypropylene	0.95 g/cm^3	4.41× 10−8 g	5.00 g/kg

Because the density of plastic in oysters is far below lethal levels, it is strongly suggested that the microplastic content in oysters has no impact on human health. If a 50 kg adult ingests 40 g of nylon 6 or 250 g of PE or PP, the microplastics will not reach the lethal level. In other words, if adults consume at least 2.52×10^8 oysters amounts to LD50. In terms of quantity, humans cannot consume that many oysters.

5.2 Standardization of the analytic methods for microplastics

Correctly analyzing microplastics in the environment is an important aspect of understanding their impacts. A major challenge in this field is the need to standardize the analytic methods for detecting and measuring microplastics. The Japanese government (Ministry of the Environment) promulgated the River Microplastic Survey Guidelines in June 2021 [31] to provide microplastic collection, preservation, and analysis guidelines. According to the guideline, the study solutions should be filtered and then treated with 30 % H_2O_2 solution for digestion at 50ºC for 24 h. The dried microplastics should be identified using Fourier Transform Infrared Spectroscopy. At the same time, the Ministry of the Environment is encouraging a worldwide horizontal distribution mapping of microplastic densities on the ocean surface [32]. Several studies are conducting similar efforts. A meta-analysis of 68 studies found that selective, volume-reduced, and bulk sampling were the main sampling strategies, whereas density separation, filtration, sieving, and visual sorting of microplastics were commonly used to process the samples, followed by the identification and quantification of the fibres found [33]. Therefore, this research proposes the analysis method for microplastics in marine life to evaluate their influence on marine life.

While the Japanese government and previous studies may have proposed a standard procedure for digesting and observing microplastics, there are several reasons why developing a new procedure may still be necessary. Firstly, we compared the different methods for studying microplastics in organisms and water. We found that KOH digestion was the most convenient. Secondly, our experimental method is not limited to filtering microplastics from water but also allows for the extraction of microplastics from organisms. In comparison to large conventional equipment such as microscopes and Raman spectroscopy, our portable developed portable digital microscope is more convenient to carry and can be easily disseminated to the general public.

5.3 Current policy of plastic production

The governments of various countries have formulated related policies because of the social impact of microplastics. Some policies are extremely effective. Some policies are difficult to implement.

Public health is relatively backward in African countries. Policies to control microplastics are also being implemented [34]. First, policies to combat microplastic pollution are difficult to implement in a single sector. Policies require multiparty cooperation among all participants in the plastic value chain and scientific researchers [35]. Thirty four African countries have implemented full and partial bans on single-use plastics [36]. All Southern African Development Community members, including 16 countries, have released plastic bag ban policies [37]—the Southern African Development Community taxes plastic bags [37]. In terms of technology, it is also one of the most efficient ways for relevant departments to improve plastic waste and further recycling [38]. Despite strong government consultations, plastic producers cite potential profit losses as the reason. Businesspeople have refused to comply with the government's plastic restrictions [39].

The Southeast Asian country (Malaysia), like some African countries, also imposes a tax on plastic bags. This policy is only enforced on Saturdays [40]. Inventing alternatives is much more welcome than charging for plastic bags [41]. In 2002 and 2005, the Indian government banned the use of plastic bags. In 2009, Hong Kong also introduced a tax on plastic bags [42].

However, the EU's political and economic decision-making appears more effective. The EU has developed a strategy from product design to cover the entire post-consumption and end-of-life process by stimulating regional recycling capacity and ensuring demand for recycled plastics [43]. Plastic waste is more widely used as a solid recovery fuel in cement kilns than in municipal solid waste incineration [44].

In China, in response to the problem of plastic pollution, the Chinese government issued "Opinions on Further Strengthening Plastic Pollution Control" in January 2020. The government formulates laws to actively promote plastic substitutes, improve the standards of plastic substitutes, and cultivate consumers' green consumption concepts through daily education [45]. In addition, the Chinese government recently implemented a new policy prohibiting most plastic waste imports [46]. Policies on plastics are widely applied in both developing and developed countries.

Japan has no microplastic pollution when compared to other countries. It may be because of the Japanese government's strong environmental protection awareness. Despite implementing the plastic bag payment policy in Japan, Japan's research on biodegradable polymers and plastics are constantly innovating [47]. Japanese researchers investigated polylactic acid and polyhydroxyalkanoates (as the most potential substitutes) [48]. Using biobased plastics produced from renewable resources and biodegradable plastics can help us solve global environmental and waste management issues [49]. Microplastics are not a serious problem in Japan because of technological advances and increased environmental awareness.

5.4 Recommendation

Although countries worldwide have effectively prevented the accumulation of microplastics by enacting laws, the best way to approach microplastic pollution should be to start from the source of plastics. The government should foster a favourable scientific research environment for scientists and invest funds in research of degradable packaging materials. At the same time, several methods, such as increasing taxes and charging plastic bags, remain necessary. Consumers should be encouraged to use reusable shopping bags, while public units should strive to promote the attitude of environmental protection and raise awareness of decreasing plastic waste in school education and daily community activities. It is crucial to be aware of the potential environmental problems caused by microplastics despite the data in this study showing that the amount of microplastics is far below the lethal amount.

5.5 References

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