深海から動物プランクトン消化管へのマイクロプラスチックスの経路

Doctoral Dissertation

THE JOURNEY OF MICROPLASTICS FROM THE DEPTH OF THE SEA TO THE GUT OF ZOOPLANKTON

September 2022

Doctoral Course of Applied Marine Environmental Studies

Graduate School of Marine Science and Technology

Tokyo University of Marine Science and Technology

RIDVAN KAAN GÜRSES

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Preface

Plastic pollution is a global pollution that increases day by day and affects more and more creatures. Unlike other types of pollution, plastics do not dissolve *per se* in nature, but continue to be a burden *in natura* only by crumbling. It gains the ability to spread further in both the hydrosphere and the biosphere by crumbling. In the present dissertation, the journey of plastic pollution from the ocean depths to the zooplankton guts was traced. The dissertation, which was created with six chapters in total, continued in the form of an inverted pyramid in terms of scope.

In the first chapter, the plastic material was scrutinized together with the significant materials used throughout history. Thus, the role and importance of plastic in the development of humanity were discussed. In addition, the distribution, spread, and transport of pollution caused by the uncontrolled (or mismanagement) release of plastic into nature was explained in detail in the atmospheric, terrestrial, aquatic, environment, and biosphere. In Chapter II, traces of plastic pollution were searched in the vertical water column sampling using MOCNESS in the Sea of Japan. Chapter III discussed the impact of structures such as marine snow on the journey to the depth of plastics in the water column. Plastic was searched in the guts of zooplankton in Chapter IV. In Chapter V, how many plastics a zooplankter can encapsulate in its gut was determined by a novel method. Finally, in Chapter VI, the general conclusion and perspectives were reflected.

In addition, while conveying this journey of plastic, the importance of the surface area of the particles was mentioned. In fact, the goal in the study was based on the challenge of explaining why the surface area of plastics is important.

The Journey of Microplastics from the Depth of the Sea
to the Gut of Zooplankton

A Dissertation
by
Rıdvan Kaan GÜRSES

Abstract

Since the 1950s, when plastic material was widely included in the market, both the production amount and product variety have increased and enriched. As a result, plastic materials have become an indispensable part of today's lifestyle, with the effect of this increased production and development. The fact that plastic material production is easier and cheaper than recycling encourages the regeneration of plastic material. Thus, constantly newly produced plastic accumulates *in natura*. The difference between plastic accumulation and the accumulation of other pollutants is that plastics cannot dissolve *per se in natura*. Therefore, the volume and scope of plastic pollution are increasing day by day. Another reason that makes this pollution even more serious is the crumbling of plastics, especially from reasons such as UV. Due to this crumbling, regardless of the initial production size, the dimensions of the product may decrease to μm levels over time. Thus, plastic particles can both move more actively and travel more easily inside the food web via the effect of this crumbling. In addition, this crumbling leads to another problem, "increase in surface area." The plastic material itself is not directly harmful, but the additives in its production and the chemicals attached to it from the environment are harmful to organisms. Exactly for this reason, the present study focused on the issue of surface area in the relationship of plastic particles to zooplankton.

The present study, which is presented in six chapters, was built as an inverted pyramid in scope. Although each of these chapters is independent in itself, it also progresses in a successive scenario. The objectives of the present study were (i) observation of plastic pollution amount in the water column, (ii) detection of plastic contamination in zooplankton in the water column, (iii) possible role of marine snow in vertical transport of plastic, (iv) plastic encapsulation capacity of a zooplankter, and (v) the surface area of plastic material encapsulated by zooplankter. In order to achieve these objectives, the six chapters were created as follows.

In the Chapter I, the importance of the material in the development of humanity was stated. How civilizations depend on materials and their periods were conveyed when these were specified. From the stone age to today's silicon age, it has been shown how materials have transformed humanity and how each new material opens new horizons. In this development story, the discovery of plastic and how it has taken over the world until today are shown. Despite all the advantages of plastic, its effects and harms on nature are reflected in a broad perspective. Contamination of plastic to the aquatic system, its distribution and its pressure on the ecosystem were discussed.

In the Chapter II, the observation of plastic pollution in the water column was examined. The samples were collected using a Multiple Opening/Closing Net and Environmental Sensing System (hereinafter MOCNESS) at the Sea of Japan with three stations via T/V Shinyo-Maru (Tokyo University of Marine Science and Technology). The maximum depths of these Stations (hereinafter Stn.)1, 2, and 3 were 100, 400, and 900 m, respectively. Samplings were made by using a 64 μm net instead of a 330 μm net, which is mostly used in plastic studies in aquatic systems. Then, micro-Fourier transform infrared spectroscopy analyzes were performed to determine the type and concentration of plastics from the samples. For example, a total of 280 particles m^{-3} were found at a depth of 500–900 m, the deepest sample of Stn. 3. The dimensioning of plastic particles found and identified were determined with image software. The surface area of the concentration of plastic due to the depth was calculated by calculating the Equivalent Spherical Diameter with the particle dimensions obtained.

The Chapter III was born from the analysis results of Chapter II. It was stated that 64 μm nets were used during the sampling with MOCNESS. However, most of the plastic samples found were less than 64 μm. For example, 1160 particles m^{-3} were found at the same station and the same depth (500–900 m in Stn. 3), less than 64 μm. Therefore, although it is difficult to reach a definite judgment, my hypothesis is marine snow formation. Therefore, the marine snow formations caught during the sampling were probably plastic-contaminated. In this way, they were involved in sampling via marine snow greater than 64 μm. In addition, the photographs of the suspected marine snow structures obtained from the samples were supported by the hypothesis.

In the Chapter IV, the effect of plastic contamination on zooplankton was studied in the same samples. First, the sub-sampling of the samples reflecting the volume of 10% was

divided. Then, all zooplankton individuals who maintain body integrity were examined from these sub-samples. The gut content of members of all zooplankton whose visible gut was controlled by a digital microscope. Particles with plastic suspicion were searched directly in the gut content. When there was a suspicious particle in any individual's gut, the body surface was wholly inspected and washed until it was sure that there was no contamination on it and was re-checked by a digital microscope. Then, plastic identification analysis was performed on suspicious individuals with the same method and instrument as in Chapter II. However, no plastic contamination was found zooplankter. Then, the method was changed, and this time only the body surfaces of the individuals were controlled by plastic identification analysis. However, no plastic-contaminated zooplankter was found again.

The Chapter V was designed as a laboratory experiment, apart from the field sampling. Although I could not find plastic-contaminated zooplankter in the field study, it is a known fact that zooplankton ingest plastic. Considering the increasing plastic pollution, the present chapter studied how many plastics a zooplankter can encapsulate. *Daphnia magna*, a model zooplankton species, was chosen for the study. Polystyrene spheres of 10 μm diameter were used as plastic. After the experiment, the gut volume was measured with a digital microscope. Subsequently, the approximate number of particles in the gut was determined using the Kepler conjecture. The surface area of the plastic particles encapsulated was calculated in the diet range, suggesting that the total surface area of plastic particles in a *Daphnia magna* gut may reach around as much as that of two soccer balls.

In the Chapter VI, the importance of the surface areas of plastics was emphasized with the findings of the thesis. The surface area should not be ignored in studies in the water column and directly on zooplankton. The surface area should not be ignored in studies in the water column and directly on zooplankton. The surface area is of great importance in transmitting both the additives of the plastic and the harmful chemicals that adhere to the plastic from the environment to the organisms.

The perspective on plastic is that we cannot prevent chemicals from the environment from sticking to the plastic, but we can limit the additives used in plastic production. For this issue, of course, additives that provide structural strengthening cannot be prevented, but additives added for cosmetics can be limited.

The perspective on detecting plastic-contaminated zooplankton should focus on a technology that can marry the μ-FTIR system with field-usable instruments such as Underwater Vision Profiler. Zooplankter gut content can be detected instantly *in situ* with such an instrument.

CHAPTER I: Introduction: Historical view of plastic pollution

Preface

Some animals have learned to shape their environment according to their needs in the course of evolution. For example, some birds were able to build nests out of bushes, and otters were able to build dams. However, the *Homo sapiens* outreach dominated the earth. They had become the only species that could move the fastest, reach the highest altitude, dive to the deepest point of the oceans, and even venture out of the planet using materials on the earth. The present chapter explains the impact of plastic pollution on humanity and the environment from a historical perspective. Hence, it describes the journey of learning and applying the materials used to establish civilization in the rise of humanity. First, how materials have changed humanity throughout history is briefly mentioned from the stone age to the iron age. Then, the use of lead by the Roman Empire and its effects on human health and the environment were mentioned. Next, the role of lead in the Romans and the role of plastic today were compared. Then, the dimensions of plastic pollution in biotic and abiotic systems were examined.

Introduction

Environmental pollution

Environmental science is a complex science that encompasses atmospheric, aquatic, and terrestrial systems (Khopkar, 2007). Humanity, which has just joined these three formations as a fourth, affects the other three with its increasing population. This effect is often referred to as environmental pollution.

Environmental pollution is a term that is constantly mentioned but not fully defined. The content of this term, whose boundaries have not been fully determined, is constantly changing and renewed. Throughout history, it was not even clear what caused the pollution. As there was no common agreement, there were also differences in definition between ages and cultures. For example, the Thames was used as sewage (Whittock, 2013) in the Middle Ages. Meanwhile, polluting mountaintops and the law prohibited flowing waters in the Asian steppes (Lane, 2006). The limits of the tolerable amount of pollution are still unknown. Alt-

hough today's world has become globalized, a consensus on pollution term has not been reached yet.

Moreover, thanks to technological developments, we both create new pollutants and discover pollutants that we are not aware of. For instance, thanks to technology and science, chlorofluorocarbons (CFCs) gases were discovered and used. Then it turned out that CFCs gases damaged the ozone layer (Molina and Rowland, 1974). The use of CFCs and other ozone-depleting substances (ODSs) was banned under the Montreal Protocol in the following years. This is just a small example, which is adorned with such cases in history.

In addition to the anthropological effect, natural effects such as volcanic activities can also cause environmental pollution. However, effects such as volcanic activities can also create new living conditions and opportunities in the long term. Therefore, there is no simple definition of environmental pollution.

Considering these reasons, perhaps the most comprehensive definition is “something in the wrong place at the wrong time in the wrong quantity” (Holdgate, 1971). Although this explanation is not very clear, it does not fall short of explaining the pollution issue. Using such a comprehensive term makes it easier to classify pollutants that will be encountered in the future.

Humanity and materials

Historians divide the that part of humanity into some criteria. These criteria are what we have or can have as simply. Human history, which is short compared to the history of the world and evolution, but which is ours, started with the hunter-gatherer period (Hill et al., 2011). During this period, people led a mobile life in small groups. This period, which is completely dependent on nature, is the way people use their environment directly. Humanity has spent all this time trying to survive. Humanity, which is constantly on the move with the hope of finding food, has also obtained the hunting tools they need from nature (Renfrew, 2008).

The period in which until writing investigation is called the prehistoric period. Prehistoric human life is interpreted with found objects (Wilson, 1851). Humans began to separate themselves from other animals with the tools they made with stones, and thus the period called the stone age began. People who showed their tool-using skills in the use of stones for the first time succeeded in making various cutting tools by using the stones around them. This period

is considered to have started with the 3.3-million-year-old stone tool found in Lake Turkana (Harmand et al., 2015).

The hominids' use of stones enabled them to learn the control of fire 1.7 to 2 million years ago, one of the most significant milestones in their history (James et al., 1989). The fact that fire control was in the hands of humanity provided an advantage against other animals in terms of hunting and defense. In addition to physical strength, fire also facilitated the digestion of food, thus saving time in daily life (Wrangham et al., 1999). Another effect of fire on food is the bacterial load, which decreases with cooking. Thus, the quality of life of humanity, which is fed in a safer way, has increased (Gowlett, 2016). Besides the direct effects as like mentioned, there were also indirect effects. According to some anthropologists, social developments occurred with the domination of fire by the hominids. It has helped a lot in the development of languages and co-working skills. It allowed people gathered around the fire to spend longer periods of time together, both during the preparation of food and for protection (Pyne, 1995). In fact, intergenerational interaction, called the “grandmother” hypothesis, is associated with the presence of fire (Hawkes, 2004). Humanity, living together and yet nomadic, has reached wide spreads where different groups can cooperate. Thus, common languages and religions emerged. Even though they were nomads, they started to build temples during this period. The oldest known example is “Göbekli Tepe,” whose radiocarbon dated to the late 10th millennium calBC (Dietrich et al., 2013). Such collaborations paved the way for the development of humanity.

In addition, global climate change occurred at the same time. During the Pleistocene period, the earth's water was mostly ice. Due to the low humidity, most of the flora consisted of tundra. Then, with the increase in temperature and the melting of the ice, the tundra left its place to the forests. As the glaciers melted, ocean currents began to accelerate (Broecker and Denton, 1990). Thus began the Holocene, which we are still in. With the onset of the Holocene epoch and the readiness of humanity's evolution, have may agriculture began (Richerson et al., 2001). Agriculture has changed the way of life of humanity. The sedentism was started to wait for the growing processes of the crops. With the cultivation of some animals, farm life began in a very similar way to today's (Ivanova et al., 2018).

The control of fire and the sedentism of agriculture enabled us to shape our environment according to our needs. Due to these needs, more robust and durable tools were needed. This quest introduced humanity to metals. Although metals are not difficult to find elements of our

planet, different temperatures should be applied according to their types for the separation of metals from the ore. The use of metals began with the smelting of copper between 5000-3500 BC. The melting temperature of copper is below 1100 °C, which made it a priority over other metals. Bronze, which emerged with the use of copper with tin, gave its name to this new civilization (McClellan III and Dorn, 2015). Although strong and durable tools could not be made from the bronze metal obtained, it led to the development of metalworking.

The development and consolidation of bronze production technology allowed the production of new metals. The development of metal technology allowed ores to be processed at higher temperatures. The iron with a melting temperature of 1539 °C has reached the point where it can be melted. Thus, iron, the second most abundant metal on our planet, began to be used in 2000 BC (Bolm, 2009).

All these developments are directly related to the materials. The dictionary equivalent of material has been interpreted as "a physical substance from which things can be made" (Stevenson, 2010). The use of materials, which started with the shaping of stones by humanity, formed the world we know. The build of cities, the formation of trade networks and the increase in population were due to the use of materials. Humanity built civilizations and destroyed other civilizations thanks to their materials.

Civilization and pollution

Humanity's learning to live together has enabled it to form collective networks. The development and expansion of the use of materials mentioned in the previous section began to establish city-states in various parts of the world. The peoples of these cities learned to live in an organized manner, and living together, thanks to the division of labor, was achieved by simultaneous playing of quite complex roles. Thus, came civilization, “an advanced level of advanced social development” (Stevenson, 2010).

Unlike the hunter-gatherer era, humanity, which started to live in complex structures such as cities, squeezed more people into the unit surface area. The sedentarism can be provided with logistics continuously and uninterruptedly in order to meet all needs throughout the year. These needs are primarily water and food. But the priority is always water, both for agriculture and for human life. Although people have established their cities close to water resources, they may be insufficient to meet the needs of the increasing population over time. The water

crisis, which could be due to reasons such as drought, was resolved by transferring water from other water sources to the cities. Various techniques were developed for the transfer of water according to the regional conditions and the source of the water. These systems can be tunnel systems such as the underground "qanat" system built by the Assyrians in the 8th century BC (Aicher, 1995). The Greek civilization took advantage of the height differences of the mountains in *Pergamum* and transferred water to the city through clay pipes with the siphon effect in the 2nd century BC (Garbrecht, 1983).

Apart from the closed systems provided by pipes, the Romans used the open system and the closed system together. The Romans, who started to carry aqueducts from long distances with Aqua Appia in 312 BC, used this system for many years and carried it to other geographies they went to. They distribute water, which was brought by aqueducts up to the vicinity of the city, to the whole city through pipes (De Kleijn, 2001). Unlike the people of *Pergamum*, who transferred water through clay pipes, the Romans used lead pipes. In fact, the origin of the word plumbing is *plumbum*, which means lead in Latin. Plumbing pipes made of lead were used throughout the Roman empire over time. For example, approximately 1.2×10^9 g of lead were used in only one pump unit of the aqueducts system in *Lugdunum* (todays Lyon), one of the medium-sized cities of the empire (Waldron, 1973).

The number of metals known to the Romans, including lead, was only seven. The use of lead in different fields brought with it an increasing demand. Thus, lead mining was conducted in various locations of the empire from Anatolia to Britain. Due to its easy processing and easy accessibility, it was used extensively in the whole country over time (Nriagu, 1983).

The use of lead in the Roman period was not limited to the plumbing. Lead has found a wide range of uses, from household items to building materials, from nautical to cooking appliances. However most emphasized is the use of the sweetening effect of lead in the production of wine and concentrated grapes. Since sugar is unknown yet and honey is expensive, people of the period used concentrated grapes as sweetener. Boiling in wine in a lead pot provided a more sedate and sweet taste. In addition to its sweetening effect, it was also frequently used for foods due to its microbicide effect (Retief and Cilliers, 2005).

Lead, which was used quite popularly by the Romans, is actually a toxic metal. Lead, which has acute or chronic effects, can be fatal (Brunton, 2011). There are some studies confirming that public health is affected by lead, which has become an important part of daily life. Apart from direct contact, water pipes are thought to play a role in this social poisoning (Delile et

al., 2017). Especially since people in the aristocratic strata have more access to this metal, poisoning has reached more serious levels. The fact that this level of society, which directs the future, is affected by such a dangerous heavy metal has made the situation socially important. In fact, some hypotheses suggest the use of lead as the reason for the collapse of the Roman empire (Nriagu, 1983). There is also the opposite of these hypotheses. There are approaches suggesting that the use of lead in the Roman period did not cause national poisoning (Retief and Cilliers, 2005).

However, there is a fact that in this period of history, as it is said in the section where environmental pollution is mentioned, the wrong substance was in the wrong place in the wrong amount. It is assumed that the Romans used about 8×10^{10} g of lead annually (Woolley, 1984). Some of this lead is from the bones of the Romans (Moore et al., 2021). Some are in lands in distant cities they founded, such as *Londinium* (Scott et al., 2020). This environmental pollution was never even discovered by the Romans, but all the lead mining history that has been transported by atmospheric means can be seen in the Greenland glaciers (McConnell et al., 2018).

Plastics

Human history is directly related to materials, as shown in previous sections. The Stone Age, Bronze Age and Iron Age are classified entirely depending on the materials. Every material that humanity learned to use carried civilization a little further. Each new material led to the discovery of the use of another material. Although these advances were always in the interests of humanity, there were also harm to their environment and themselves, as well as the side effects of the use of lead in the Roman period. This can be explained by the principle that "every action has a reaction" (Newton's third law of motion), which is one of the fundamental laws of physics. Despite everything, the environment in which humanity lives today is built entirely with the use of materials. Civilizations have discovered and used other materials besides stones and metals. Humanity used ceramics, fibers, glasses, horns, skins (leathers), metals, stones, and woods until the nineteenth century. Everything known in this period was made with these eight materials and their combinations. In the nineteenth century, two new materials, rubber and plastic emerged, which enabled the more effective use of these eight materials and the production of new materials that were unknown before. However, natural plastics (bitumen, tree resins (amber), horn, tortoiseshell, lac, shellac, gutta percha, balata,

natural rubber, *etc.*), which are currently in the plastic class, date back to ancient times (Gilbert, 2017).

In 1820, Thomas Hancock of Marlborough produced a fluid plastic when highly sheared or masticated rubber. Then, in 1839, when American Charles Goodyear heated rubber by adding sulfur, the temperature range of its flexibility increased compared to raw. Around the same time as Goodyear, Hancock noticed the effect of sulfur. Although some researchers think that Goodyear discovered it before, Hancock patented it in 1843 (Hancock, 1857) while Goodyear patented it in 1844 (US Patent, 3462). In the last half of the same century, cellulose nitrate products called parkesine and xylonite (also ivoride) appeared, respectively, from Europe. Collodion, also a cellulose nitrate product by Hyatt, was launched across the Atlantic with its European counterparts. In the first quarter of the twentieth century, casein plastics emerged, formed by the reaction of casein, a milk protein, with formaldehyde. Formaldehyde resins have been produced close to each other with casein. In a short time, many types of phenol-aldehyde plastics entered the market in many different areas of use. In this period, cellulose acetate also found its place in the market. By the 1930s, four of today's major thermoplastics, polystyrene, polyvinyl chloride, polyolefins and polymethyl methacrylate, materialized. In 1933, Fawcett and Gibson introduced polyethylene (PE) to the market. In 1938, nylon 66, the first fully synthetic fiber patented by Du Pont, found its place in the market. Polytetrafluoro-ethylene was also found during this decade. In the 1940s, acrylonitrile butadiene styrene (ABS) terpolymer came into the field (Gilbert, 2017).

Though by the 1950s, the actual rise of plastic began. World War II has improved its techno-logical and production infrastructures. For example, after the war, plastic materials such as PS and PE were produced in tons for public use. The abundance of PS and PE plastic types, previously produced in small quantities and expensive, enabled plastics to find new uses. The war also changed the raw material of plastic. Before the war, the most important raw material of plastics was cellulosic. Then coal was used as raw material. The use of coal continued until the mid-1950s. By the 1960s, the plastic raw material turned into petroleum, which is still used today. For production, crude oil goes through fractional distillation. Thus, the petroleum gas, gasoline, naphtha, paraffin, diesel, fuel oil, lubricating oil and bitumen products appear. Among these products, ethylene (C_2H_4), the most important product for plastic, is obtained from mainly the naphtha (C_6–C_{14}) (Sarker et al., 2011). Producing plastic from ethylene can be seen as an alchemical feat. Various methods are applied to convert monomer structures such as naphtha to polymer structures such as ethylene. These methods are high-pressure,

Ziegler-Natta, Phillips, standard oil, and metallocene processes, chosen according to the product produced and the material properties obtained. After the process followed, the carbon monomer is converted into carbon-based polymers called plastics (Ronca, 2017).

Plastics are generally classified into two groups. These two groups are thermosets and thermoplastics. Thermosets (epoxide: EP, phenol-formaldehyde: PF, polyurethane: PUR, unsaturated polyester resins: UP, *etc.*) are plastic types that do not soften after solidification. On the other hand, thermoplastics (acrylonitrile butadiene styrene: ABS, polyethylene: PE, polyethylene terephthalate: PET, polytetrafluoroethylene: PTFE, polyvinyl chloride: PVC, polymethyl methacrylate: PMMA, polypropylene: PP, polystyrene: PS, *etc.*) are those that can soften again each time they are heated (Rosato et al., 2004).

Each type of plastic has different usage areas. In addition to the difference of types, the same type of plastic can find different uses with additions. For example, products such as blood bags, pressure pipes, window frames, flooring, and indoor insulation are produced from polyvinyl chloride (PVC) with different additions. Additions vary according to the desired function of the plastic. Major function classification: anti-aging, blowing agents, colorants, effect modifiers, fillers, flame retardants, flow enhancers, lubricants, plasticizers, softeners. Although some of these additions have the purpose of making the material more convenient, durable or useful, they are also used only for cosmetics, such as colorants (Al-Malaika et al., 2017).

Plastic pollution

As mentioned in the previous section, although plastics entered our lives in the 19th century, it was only in the second half of the 20th century that petroleum-based production methods produced them in high quantities. Unlike the Romans, thanks to the fact that 20th century people knew every continent of the planet they lived on and that trade routes connected these continents, plastic material spread rapidly worldwide. Thus, plastics have taken over the world faster than any other material in history the time to date. This material, which has become an important part of human life, can be found wherever humanity can reach. They are used in a wide range of areas, from the construction of the Voyager 1 satellite, which is 23×10^9 km away from earth to the insulation of the leads of pacemakers that help the human heart. Plastic is seen on the car wheel, toothbrush, mobile phone, wristwatch, the computer on

which this text is written, in short, almost everything used in daily life. Since the product is produced and used worldwide, its pollution has also become worldwide.

Plastic pollution begins in the terrestrial environment, as the producer and consumer of plastic material are *Homo sapiens*, a terrestrial organism. Today, more than half of the human population lives away from the ocean coasts, while 3 billion live within 200 km of the coasts (Creel, 2003). Although plastics are recyclable types, recycling is not yet the preferred way for plastic material. As such, almost every plastic product produced ends up being garbage (Nizzetto et al., 2016). As of today, the most preferred method is landfill, that is, to surrender the plastics to their fate. In this way, plastics are dispersed into the environment by leaving the garbage collection areas by rain, wind, or animals. However, not all plastics can collect as garbage and spread to the environment out of control.

Considering that this amount is up to 23 times more than the amount of plastic in the oceans annually (Horton et al., 2017), it can be understood how significant the accumulation is in the terrestrial ecosystem. Plastic pollution in the terrestrial environment is not only caused by single-use plastics. There are 1-60 particles m^{-3} of plastic, most of which are microfiber, in indoor environments (Dris et al., 2017). These fibers, mostly dispersed from synthetic textile products, also release up to 7×10^5 fibers into the water in each wash with the washing machine (Napper and Thompson, 2016). In addition, there are plastics in beads form in the content of some personal care products. These beads can be included in various products such as skin creams, peeling products, shampoos, shower gels, toothpaste. For example, from using this type of toothpaste, 8.7×10^8 g of plastic beads per year can spread to the environment in İstanbul (Ustabaşı and Baysal, 2019).

Plastics are also contained in non-aqueous paints. Due to the corrosion of paints over time, they spread around as particles. These paint types are used to cover surfaces such as buildings and metals. It is a preferred type of paint for its water resistance, resistance to degradation, and even for its aesthetic appearance. Also, this type of paint is used for road marking projects due to this resistant structure. It is estimated that 5×10^8 g of plastic particles are emitted annually from the paint used for road marking in Sweden (Magnusson et al., 2016). Nor are they the only plastic source markings on the roads. In addition, the parts of the vehicles using the roads due to the wear of the tires are also a source of plastic pollution. This contribution of tires has been calculated as yearly approximately 8×10^2 g per capita as global (Kole et al., 2017).

On the other hand, agriculture is another plastic consumer and pollutant. The increase in the global population means that the need for food also increases. As a result, the agricultural sector began to use more and more techniques and land. Among these techniques are the use of mulch film to conserve water in the soil, and the greenhouse technique, which allows vegetables or fruits to be produced out of season, is also applied using plastic materials. In 2017, only China used more than 1.4×10^6 g plastic mulch film material (Batool and Qadir, 2022).

Another important source of plastic pollution is the industrial factor. The fact that the material is produced at this stage carries this ring to an important position. Plastic materials are produced as beads due to their ease of transport and storage. During the transportation of materials, there are accidental leaks to the environment (Antunes et al., 2018). Likewise, when processing methods such as cutting the material are applied, small particles may be dispersed into the environment. In addition, the spread of plastic resins or powders used in such as air blasting machines to the environment is another pollution ring (Gregory, 1996).

In the previous sections, lead particles emitted from the lead mines and processing of the Romans and reaching as far as Greenland was mentioned. Another example was the Godzilla dust storm in June 2020. The Gulf Coasts of the USA were affected in this event, where sand rising from the Sahara Desert was with airflow across the Atlantic Ocean. It is known that plastics can similarly be transported atmospherically. Plastics emitted into the environment from various sources, such as powders or resins from air blasting machines, paint particles, clothes fibers, and car tires particles, are contained in city dust. For example, 88 to 605 particles of plastic were found in 30 g of dry city dust in Tehran (Dehghani et al., 2017). While there is more plastic in dust content in industrial areas than in urbans (Abbasi et al., 2017), there is a higher amount of plastic in buildings compared to cities (Dris et al., 2017). The plastics contained in the city dust structure are caught in the air circulation and are transported with airflows. For example, it had found that plastics move with the airflow in the study (365 plastic particles$\cdot m^{-2}day^{-1}$) carried out at the Bernadouze meteorology station located in the Pyrenees Mountains (Allen et al., 2019). Another example is the assumption that plastic particles found in snow samples taken from areas close to the summit of Mount Everest come by atmospheric fallout (Napper et al., 2020). Plastics circulating with atmospheric fallout, both in cities and rural areas, can be transported to aquatic systems (Verschoor et al., 2016).

As briefly mentioned in the section "civilization and pollution," civilizations are directly related to water resources. This situation has not changed since ancient times due to the water need of humans. There are hundreds of examples, such as the Osaka established around the Yodo River, or the Rome established around the Tiber River. The amount and types of plastic vary depending on the socio-economic or cultural differences of the population around these water sources. For example, while plastics originating from personal care products cause environmental contamination in affluent districts, plastic particles and Zn contamination originating from tires occur in areas with heavy traffic. Likewise, mulch film particles are denser around agriculture fields, while suburban areas have more clothing fibers (Lambert and Wagner, 2018). Both the plastic particles in the dust in the air and the ones on the ground are carried to these rivers with precipitation. Every year, up to 2.41×10^{12} g of plastic is discharged from the world's river systems into the oceans. For example, the Danube River transfers 1.5×10^{9} g of plastic annually to the Black Sea in the European continent. On the other hand, studies conducted in Chesapeake Bay in North America have found an intense amount of plastic (Lebreton et al., 2017). Although every river connected to the seas worldwide is a source of ocean plastic, only 10 rivers are the source of 90% of all ocean plastic (Schmidt et al., 2017). Of these top 10 rivers, one river carries an incomparably more significant plastic load to the oceans than any other river. The Yangtze River, which rises in the Tibetan plateau and flows into the East China Sea after a long road, takes the first place by bringing 3.3×10^{11} g of plastic annually to the ocean system. The Ganges River, which rises on the other side of the Himalayas, passes through India and Bangladesh and flows into the Bay of Bengal, ranking second with 1.2×10^{11} g per year (Lebreton et al., 2017). These plastics, which are constantly flowing into the oceans, accumulate in the oceanic system.

Plastics entering the ocean system, whether from rivers, the atmosphere, or shipping, are carried along with ocean currents. Today, a total of 25×10^{10} g of plastic floats on the ocean surface (Jamieson et al., 2019). Some drifting plastics gets caught by ocean gyres. There are ocean gyres at 5 points in the world's oceans: North Pacific, South Pacific, North Atlantic, South Atlantic, and Indian Oceans. In the chapter of the Sargasso Sea in Jules Verne's novel Twenty Thousand Leagues Under the Sea, published in 1870, he described how floating debris accumulates in ocean gyres. Since Verne's book was published in 1870, the floating debris in the oceans still exists, but its contents have changed with the addition of plastics. In fact, the North Pacific Subtropical gyre gained the name of Great Pacific Garbage Patch due to its plastic issue. The weight of the plastic dragged by this gyre alone is 8×10^{10} g, the same

weight as the lead produced by the Roman Empire per year (Lebreton et al., 2018). On the other hand, plastics drifted by ocean currents can be transported far from their pollutant source. The famous Moby Duck story shows how plastics can be dispersed by ocean currents when rubber ducks accidentally spilled into the Pacific Ocean on a ship carrying cargo from China to the USA in 1992, crossing the Arctic Ocean and hitting Europe's Atlantic coast 2007 (Boxall, 2009). Moreover, plastic pollution is not just an issue of the ocean surface. They can also move vertically in the water column. It is even assumed that up to half the volume of plastic that has entered the ocean system has sunk (Kaandorp et al., 2020). These plastics, separated from the surface water and moving into the depths, both become a part of the water column and are included in benthic zone (Song et al., 2018). The plastics have even reached the Mariana trench, known as the deepest point on the earth (Jamieson et al., 2019).

The most critical factor that distinguishes plastic from other pollutants is that it does not dissolve *per se in natura*. Plastics, the immortal material of the mortal world, only crumble with some effects. These effects are radiation (solar, nuclear, thermal), temperature (elevated, depressed, cyclic), water (solid(snow, ice), liquid (rain, condensation, standing water), vapor), normal air constituents (oxygen, ozone, carbon dioxide), air contaminants, gases (oxides of nitrogen and sulfur), mist (aerosols, salt, acids, and alkalies dissolved), biological factors (fungi, bacteria), stress factors (sustained, periodic (physical action rain, hail, sleet, and snow), the physical action of wind, movement due to other factors, incompatibility factors (chemical, physical), use factors (design of system, installation and maintenance procedures, normal wear and tear, abuse by the user), and particulates (dirty, sand, dust) (Gray et al., 1999). The sizes of plastics, which only break and shrink with these effects, have become important for research over time and have been classified according to their sizes. Small size plastics were first named microplastics (µPs) by Thompson et al. (2004). Presently afterward, it was limited to the upper limit of 5 mm. Then next, µPs were divided according to their origin into primary (manufactured to microscopic dimensions) or secondary (resulting from environmental degradation and degradation processes). The growing interest in the subject led to the need for classification in smaller particles than µPs. Thus, the lower limit for µPs has been determined as 1 nm (Frias and Nash, 2019).

Plastics in the aquatic food web and potential effects

The lithosphere, atmosphere, and hydrosphere contaminations of plastic pollution were described in previous sections. The last remaining major subsystem, the biosphere, has also been affected by plastic pollution. The relation of plastic material with the biosphere is still an issue that has not been fully elucidated. The first reporting for ingestion was noticed in the Laysan albatrosses (*Phoebastria immutabilis*) sampled in the Northern Hawaiian Islands in the late 1960s (Kenyon and Kridler, 1969). Following this report, similar reports were published from different parts of the world. For example, seabirds such as stranded prions (*Pachyptila* spp.) from New Zealand and Leach's storm petrels (*Oceanodroma leucorhoa*) from Canada have also been reported to have plastic in their stomachs. On similar dates, it was noticed that seabirds and other sea animals are at risk from plastic. In the 1950s, it was noticed that marine turtle's ingestion of plastic bags. By the mid-1970s, also cetacean species were reported to ingest plastic products such as fishing gear (Ryan, 2015a). Once called a revolutionary material, plastics started to affect more marine animals and show themselves more in the aquatic system over time. Hence, with the help of the public interest, it became a subject of study worldwide in the first quarter of the 21st century. Thus, plastic contamination has been observed in animals at different trophic levels, such as amphipods, annelids, aves, barnacles, bivalves, cephalopods, cetaceans, cnidaria, decapods, echinoderms, fishes, isopods, protists, reptiles, and various zooplankton (Lusher et al., 2017b).

Although it is known that plastic pollution affects different trophic levels, there is no clear view of its harms yet. The more research is done on the harmful effects of plastics, the better their short- and long-term effects can be recognized. However, it is possible to assume potential effects from the material's content. The effects of plastic on organisms are examined in two main categories. One of these categories, the physical effect, is related to the plastic's size, shape, and amount. The other is the chemical effect, which is more complex than the former. Plastics contain two types of chemicals separated by their origin. As mentioned before, while producing plastics, some additives are used that create the desired properties. In addition to this chemical group, there are also chemicals that plastics adsorb from their environment (Campanale et al., 2020).

An effect or material that disrupts the working system of a cell, organ, or organism is called a toxin. Chemical toxins are harmful to the nervous system, internal organs, reproductive system, and even DNA (cause of cancer mutations). Another effect is on the endocrine system.

Some chemicals can trick the endocrine system. This situation can lead to hormonal imbalances; chemicals act like hormones and send the wrong message to the system. These endocrine-disrupting chemicals (EDCs) can cause reproductive disorders, metabolic disorders (obesity, diabetes), asthma, cancers (testes, breast, prostate), and neurodevelopmental conditions (Cingotti and Jensen, 2019).

Bisphenol A (hereinafter BPA) is a carbon-based synthetic compounder, which is a common plasticizer used in the production process of polycarbonate plastics and food packaging products. Since it is used in plastics in direct contact with food, its presence is more careful about humankind. However, BPA is associated with breast cancer, cardiovascular disease, reproductive disorder, and obesity (Campanale et al., 2020). It also has harmful effects on aquatic organisms. It has been reported that BPA affects the reproductive system (decrease sperm quality, inhibition of spermatogenesis and egg) in non-mammalian vertebrates and disrupts the sex ratio of the population. According to the United States Environmental Protection Agency (USEPA) reports, BPA showed high chronic aquatic toxicity. However, in invertebrate species, the exposure time was not determined precisely in the study conducted with daphnids (Canesi and Fabbri, 2015). In addition, in a study with *Daphnia magna*, BPA intake and body accumulation were observed (Rehse et al., 2018).

Another potential toxin additive is phthalates. These types of chemicals are included in various plastic products such as insecticides, household furnishings, cleaning materials, clothing, nutritional supplements, dentures, children's toys, food packaging, automobiles, lubricants, waxes, cosmetics, building materials medical devices, pharmaceuticals. These are chemicals added to the basic plastic to give modified properties such as flexibility and elasticity (Campanale et al., 2020). Members of this chemical family are di-ethyl-phthalate (DEP), butyl benzyl phthalate BBP, di(2-ethylhexyl) phthalate (DEHP), di-*n*-hexyl-phthalate (DnHP), di-*n*-butyl phthalate (DBP), di-*n*-octyl-phthalate (DnOP), di-isononyl phthalate (DINP), dimethyl phthalate (DMP), di-isodecyl phthalate (DIDP). Each of these chemical additives has different uses for different products. Some types are prohibited, or the dosage is limited. These chemicals, which exhibit low acute toxicity, have harmful effects on the liver, kidney, thyroid gland tissue, and testicles (Heudorf et al., 2007). There are not many studies yet on its effects in the aquatic ecosystem. However, Pietrini et al. (2022) found that DMP had genotoxic and physiotoxic effects on model plants such as *Lemna minor* L. and *Spirodela polyrhiza* (L.) Schleid.

In addition, another additive is heavy metals. Unlike other additives, heavy metals are part of nature. They become toxic with increasing concentration due to anthropological effects. Just like the use of lead by the Romans and the health problems arising from it, there are heavy metal and related problems in plastic. Heavy metals are added to plastics for various purposes. One of these reasons is to gain effect to the biocide. Although polymers are resistant to biological attacks, some organisms can cause a degrading effect on plastics. For this reason, by adding biocide-effective metals such as arsenic (As), antimony (Sb), and tin (Sn), the product is made more resistant to biological effects. Another class of additives is heavy metals with a stabilizer role. These heavy metals are lead (Pb), cadmium (Cd), antimony trioxide (Sb_2O_3), and tin (Sn). These metals are added to provide durability against degradation by UV, oxygen, and temperature changes. Even a petroleum-based material such as plastic can be given flame retardants with heavy metals. For this purpose, antimony oxide (Sb_2O_3), zinc borate ($B_6O_{18}Zn_9$), and aluminum oxide (Al_2O_3) become a part of plastic as additives. The use of heavy metals as additives is used not only in structural reinforcements but also in cosmetic (as colorant) modifications. Metals with these additives classified as inorganic pigments are zinc (Zn), chrome (Cr), cadmium (Cd), lead (Pb), titanium (Ti), and cobalt (Co) (Campanale et al., 2020). Some of these metals (As, Pb, Hg, Cr, Cd), which create a load in plastics, cause various cancers on animals and humans (Tchounwou et al., 2012).

Toxicity is not only caused by the plastic itself, but also comes from the ambiance as mentioned at the beginning of this section. These chemicals, also called the latter chemicals, adhere to the surface of the plastics. These types of chemicals include various persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAH), polybrominated diphenyl ethers (PBDE), pesticides (Delaeter et al., 2022).

The quantity of toxins acquired from both the additives and the environment (POPs) are directly related to the surface area of the plastic. For this reason, it would not be wrong to say that as the surface area increases, the potential harm will increase (Rochman, 2015).

Composition of the present and following chapters

The thesis was created based on the relationship between planktonic organisms and plastic pollution. This relationship was examined both *in situ* and *ex situ* in different frameworks.

The present chapter explains from a historical point of view that materials are an essential issue in the development of humanity and how the pollution arising from it affects the world and humanity. The variety of uses of plastic material, the story of the pollution arising from its widespread use, and its potentially harmful effects on organisms were also discussed as side effects of the development of civilization.

In the Chapter II, the vertical distribution of plastics was examined with samples collected from different isolated depths between the surface to 900 m depth in the Sea of Japan. In addition, the Chapter III discussed the possible influence of biological factors on the descent of plastics from surface waters to depths. In Chapter IV, plastic contamination in zooplanktonic organisms in the same samples was investigated.

Finally, in the Chapter V, the plastic encapsulation capacity of individual zooplankter was found by testing it on the model zooplankton species *Daphnia magna*.

In addition, the Chapter VI concludes by summarizing all results. In addition, perspectives were presented to reduce the possible harms of plastic pollution on living things. Moreover, perspectives were presented on devices that could be developed to detect plastic contamination in zooplankton guts.

CHAPTER II: Vertical distribution of microplastics in the water column

Preface

In the Chapter I, it was mentioned how the materials have guided and developed human history. Then, pollution, which can be called the side effects of this development, and its possible effects were discussed. It was also mentioned that studies on plastic pollution, which is growing both production and pollution, are increasing rapidly. It has been shown that atmospheric and hydrospheric movements can disperse this material. It has even been shown that plastic pollution has reached both the summit of Everest and the Mariana Trench. The present chapter focused on vertical plastic distribution with three station sampling at Sea of Japan. The water column of different depths was filtrated with 64 μm mesh nets in isolation in the sampling using Multiple Opening/Closing Net Environmental Sensing System. Different ratios of plastic material were found in all analyzed depths. The results showed that plastics are a part of the water column and there are differences in size and quantity between depths.

Introduction

The statement about plastic pollution and the possible effects on the ecosystem was explained in the previous chapter.

Plastic pollution studies in the marine environment continue with increasing interest. Plastic pollution studies, which began to be reported in the 1970s, have intensified in the last two decades. As a result of increased studies, the knowledge of plastic travel in the atmosphere and hydrosphere has become more comprehensive. Although pollution studies in the water system have increased, a significant part of the studies has focused on the surface layer. Plastic pollution on surface layers has been reported in all five oceans (from the Arctic to Antarctica). In addition, there are many studies on the transport and accumulation of plastic material on the ocean surface.

On the other hand, information on the status of plastic pollution in deep pelagic water is still relatively shallow. The deeper pelagic zone is less well known than in other parts of the world, not just in plastic pollution research. This situation is because deep layer sampling is

more complex than surface water sampling. It requires more knowledge skills, technical skills, and technology. For these reasons, it is a more expensive sampling method.

However, it is important for the marine ecosystem to reveal the vertical distribution of plastic particles as well as horizontal distribution on the surface. In order to know the interaction of pelagic zone organisms with plastic, first of all, the relationship of plastic with this zone should be clarified.

In addition to the different mechanical and chemical properties of plastic types, their densities are also different. The density difference of plastics is examined by taking the density of water as the origin, as less or denser than water in the aquatic environment plastic pollution studies. For example, polyethylene (0.91–0.95), polypropylene (0.9–0.92), polystyrene (1.01–1.05), and have lower specific gravity (the ratio of material density to pure water at 4°C) than seawater (1.027), while polyvinyl chloride (1.16–1.3), poly(ethylene terephthalate) (1.34–1.39) and polyester resin (>1.35) have higher specific gravity (Lusher et al., 2017a). For this reason, some plastics directly sink when they enter the aquatic system, while others floating in surface waters.

Cózar et al. (2014) observed an increasing amount of smaller particles on the ocean surface up to a threshold of 2 mm in size. On the other hand, particle abundance of 1 mm and smallers decreased with decreasing particle size in the same study. There are various ideas about the inability to detect plastics below a certain size in surface waters. Some of these ideas show the lower limits of the instruments used in capturing and identifying plastics. However, other ideas more realistically base this event on the working principles of the aquatic system. As far as is known, plastics do not dissolve *per se* in aquatic system. Plastics are only crumbled by some effects (widely explained in the Chapter I). Thus, plastics only shrink and continue to exist in the system. This shrinkage also affects the buoyancy of plastics. It is thought that plastics sank due to the loss of buoyancy as they get smaller. Although this situation is realistic, there is a lot of evidence that the biological pump is the main cause (Kvale et al., 2020). It has been shown in several studies that zooplankton ingest microplastics (µPs) and thus become part of fecal pellets (Cole et al., 2016). Moreover, plastic materials included in the aquatic system become an object of attachment for the marine organism (bacteria, algae, crustacea, *etc.*). These organism groups (micro and macro biofouling organisms) can settle on the surface of the plastic material and affect the density and weight of the material. In addition, µPs was observed in the structure of aggregates called marine snow. The µPs can be in-

cluded in the system from any point of the biological pump structure (shown in the Figure 2.1.1.). Thus, even if the densities of µPs themselves are less dense than seawater, they can precipitate by being included in biofouling, fecal pellets, or marine snow. The results of some simulation experiments have shown that the sinking fate and the rate depend on the dimensions of positive buoyant µPs. The biofouling rate slowed down as the particle sizes increased between 1 mm and 0.1 µm dimensions in the experiment carried out by Kooi et al. (2017). In the same study, it was determined that 10 µm particles sink slowly and continuously, but 100 µm or 1 mm particles move up and down in water.

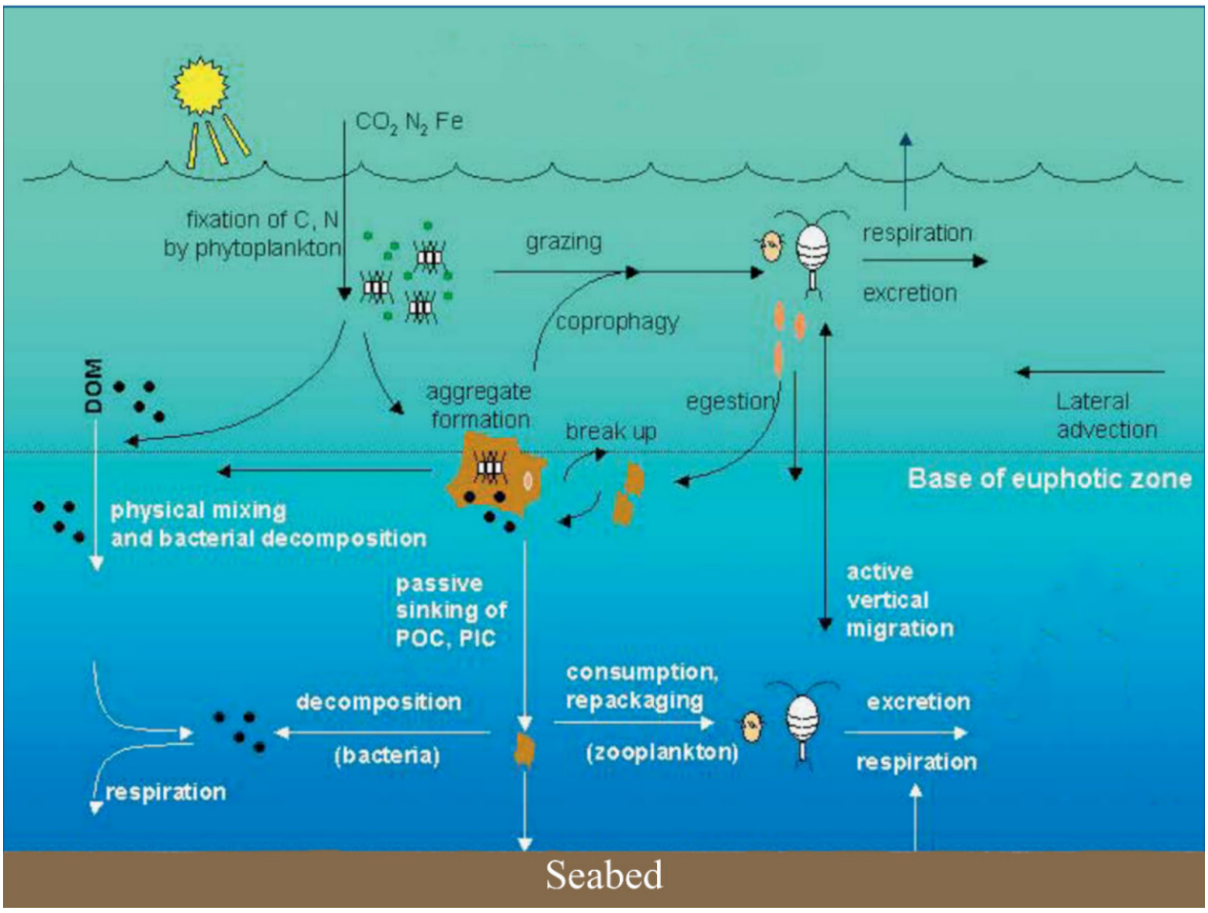


Figure 2.1.1. Schematic representation of the biological pump (Ducklow et al., 2001). Fundamentally, it is the order that allows the nutrient elements to spread in the surface waters in the aquatic system and in the sediment (where DOM: Dissolved Organic Matter, DIM: Dissolved Inorganic Matter, PIC: Particulate Inorganic Carbon, POC: Particulate Organic Carbon). Since plastics are also included in aquatic systems and are ingested by zooplankton and even participate in marine snow structure, the biological pump can guide understanding of the transport of plastic in the water column.

Plastic pollution studies in the water column are not limited to simulations. Plastic samples can be isolated from the water column by various methods. Plastic sampling can be done via the water column with either net sampling methods (such as bongo, Apstein, Nansen, Juday, WP2, multi-net trawl, multiple opening/closing net environmental sampling system, continuous plankton recorders) bulk techniques (Niskin bottles, rosette device, integrating water sampler, immersible pump), or remotely operated vehicles (Choy et al., 2019; Lai et al., 2021).

Currently, plastic pollution of the water column has been reported in various different parts of the oceans. For example, the water columns between the surface and 5 m were sampled using a multi-level trawl (MLT) equipped with 330 µm mesh nets in the study conducted by Kooi et al. (2016) in the North Atlantic Ocean. Reisser et al. (2015) also has a study in the same ocean (the North Atlantic Ocean) with the same sampling method (surface to 5 m depth with MTL) but with 150 µm mesh nets. In addition to the Atlantic Ocean, studies are conducted with various sampling tools at different points in the Peaceful (Pacific) Ocean, the largest ocean on the earth. For example, a study determines the amount of plastic by filtering (100 µm mesh) the samples taken from different depths to 1000 m from the surface by using remotely operated vehicle (ROV) on the central California coasts in the Northwest Pacific (Choy et al., 2019). Another study was conducted in the Great North Pacific Gyre (also called North Pacific Garbage Patch), widely mentioned in plastic pollution studies. Unlike the others, Multiple Opening/Closing Net and Environmental Sensing System (hereinafter MOCNESS) was used in the study conducted in North Pacific Garbage Patch (NPGP). 333 µm mesh nets were used in this study, which was carried out at 5 stations from Honolulu to Rosarito (Egger et al., 2020). More similar studies focused on water columns than described, but the important point is that plastic particles were found in all the depths examined in all the studies. It is known that the plastic load in the sea, which has been calculated by studies conducted only in surface waters, constitutes only 1% of the estimated pollutant plastic load (Cózar et al., 2014). Although sampling from the water column is more costly and requires higher technique, it is essential to understand the extent of plastic pollution.

In the present chapter, the aim was to determine the concentration of plastic pollution in the water column with vertical samples taken from three different stations. During the cruise from Hakata port to Maizuru port, samplings were made at all three stations with the MOCNESS. Unlike similar studies (µPs studies mostly use 300 µm mesh net), the MOCNESS is equipped with 64 µm mesh nets. Thus, smaller particles (< 300 µm) were also captured with

each depth sampled. Plastic material was found at all depths sampled and analyzed in the study. In addition, most of the particles found were under 300 μm in length. In addition to the studies focusing only on the size of the plastic particles, the volumes of the particles found in the present study were also discussed. Thus, it demonstrated that (i) whereabout the missing small plastic particles, (ii) plastic pollution studies should pay more attention to the water column, and (iii) volume is as important as size.

Materials and methods

Study site

The Sea of Japan is comparable to the ocean characteristically; it contains warm and cold currents, eddies, and planetary hydrological and local fronts. Therefore, it is also called the miniature ocean (Ichiye, 1984). This sea is fed by the Liman current from the north and the Tsushima current, extending the Kuroshio current from the south. In particular, the Kuroshio current, which is one of the major currents carrying the most plastic load in the world, affects the Sea of Japan (Nakajima et al., 2021). Also, this sea is surrounded by Japan, Russia, North and South Korea. For this reason, besides the plastic load carried to the region by the Tsushima current, there is also a contribution from the countries of the region.

Samples were collected with Tokyo University of Marine Science and Technology's training vessel (hereinafter T/V) Shinyo-maru in July 2018. In this cruise, which was made from the Hakata port to the Maizuru port, samples were collected at three different locations, which were Stn. 1 (34.27°N, 130.18°E), Stn. 2 (35.55°N, 131.56°E), and Stn. 3 (36.16°N, 134.42°E). The three stations are shown in Figure 2.1.1. as a map.

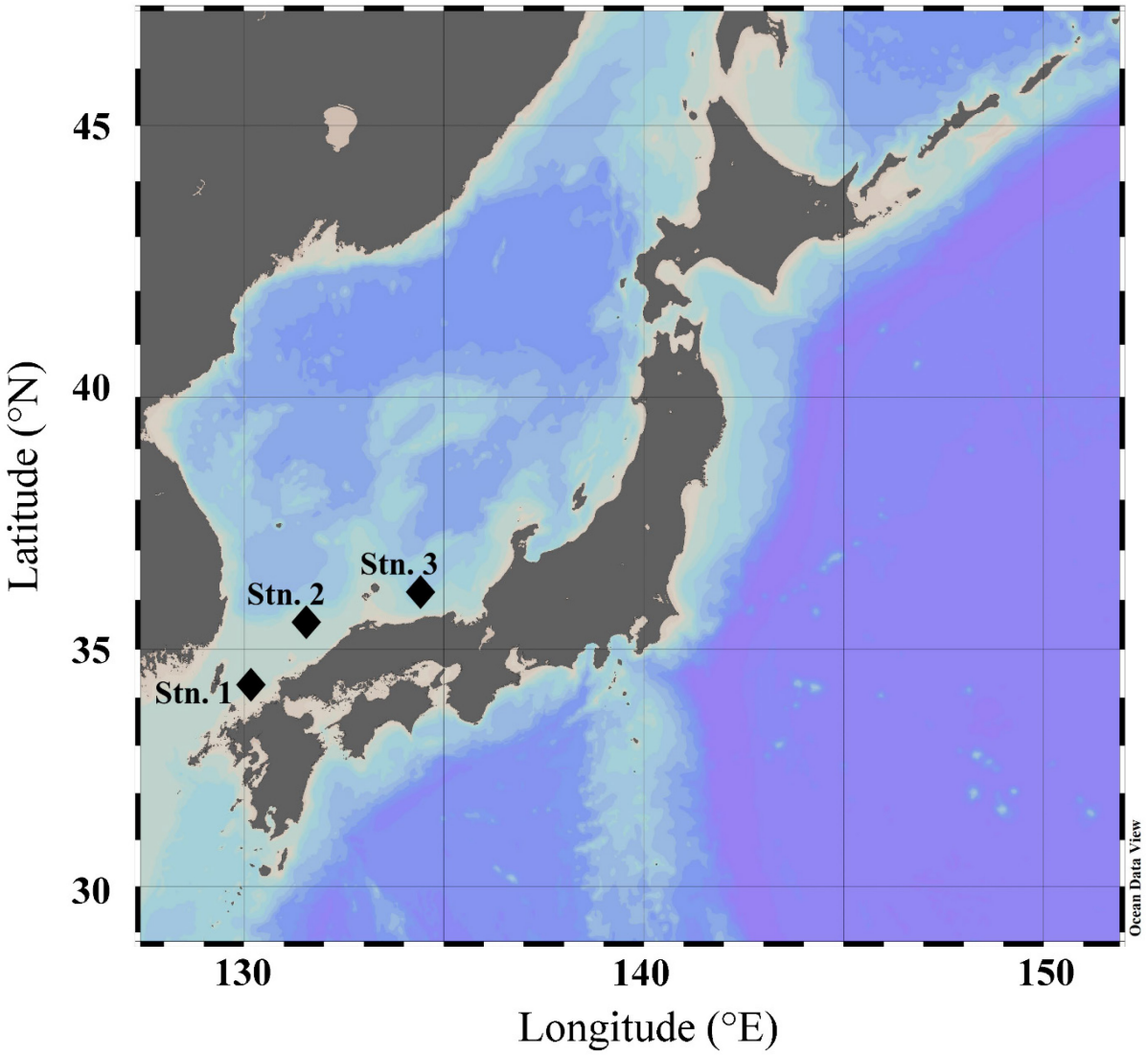


Figure 2.2.1. Sampling stations in the Sea of Japan.

Sample collection and preparation

Samples were collected at each station using MOCNESS. The principle of this system is a design based on the Tucker trawl (Wiebe et al., 1976). As with this trawl, it can be equipped with multiple nets and integrated with environmental sensors. Sensors monitor conductivity, temperature, depth, chlorophyll, oxygen, light levels, flow, and angle instantly. MOCNESS, which can dive to a depth of 6000 m, can instantly transmit sensor data to the deck. It can be equipped with 5 to 20 different nets, and the net can be changed instantly from the deck. In addition, it can be equipped with nets with mesh holes from 64 μm to 3 mm. Since each net can be activated when desired, sampling can be collected between desired depths. During the operation (Figure 2.2.2.), the system is descended into the water and dives; (i) the first net is

descended open while descending to the desired maximum depth, (ii) then, a new net is opened and the previous one is closed at each desired depth. Thereby, sampled are collected between the depths while being raised again (Wiebe et al., 1985).

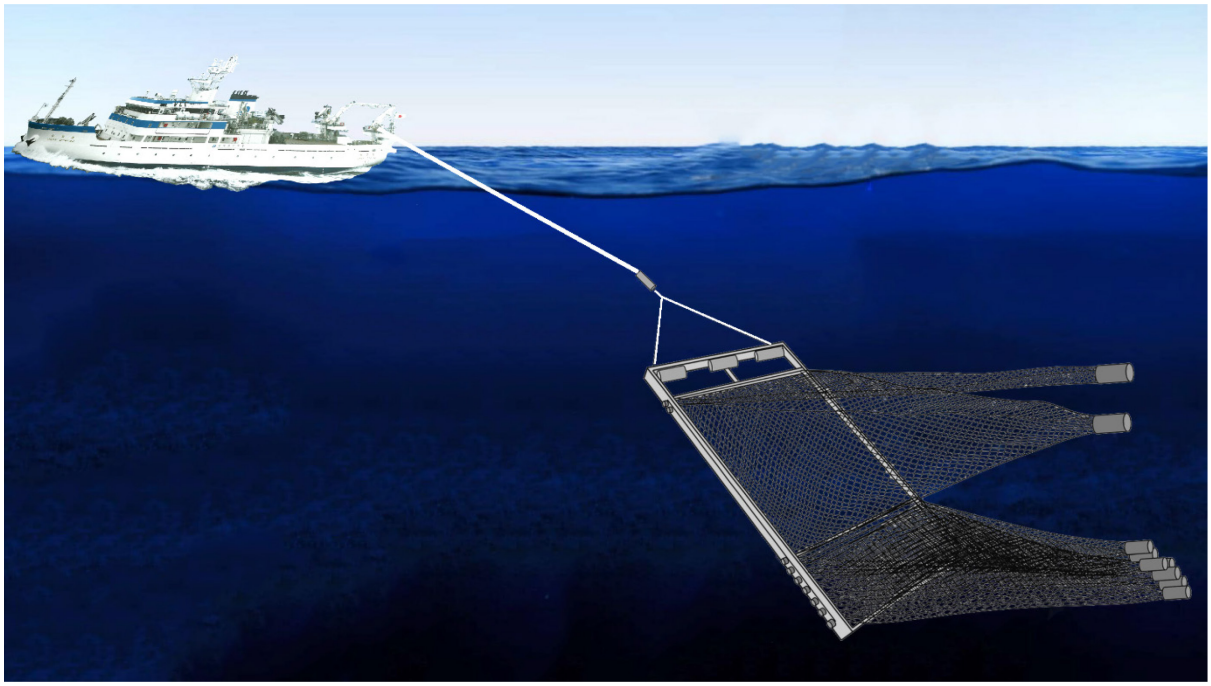


Figure 2.2.2. Schematic drawing representation of Multiple Opening/Closing Net and Environmental Sensing System (MOCNESS)

In the present study, the MOCNESS equipped with 9 nets of 64 µm was used at all three sampling stations. The maximum depths of Stn. 1, 2, and 3 are 100, 400, and 900 respectively. Samples were made from these stations from different depths. As stated above, the first net was used for diving from the surface to the maximum depth. Therefore, the column was divided by the remaining eight nets. The volume of filtered water for each depth is shown in Table 2.2.1. The speed of the vehicle was kept around 2 knots during the sampling. The filtered volumes were calculated with the flow meter integrated into the MOCNESS system.

Table 2.2.1. Filtered volume per depths of vertical water column sampling from the Sea of Japan using MOCNESS.

Stn.	Net	Depth (m)	Filtered volume (m^3)
1	1	100–75	60
	2	75–50	60
	3	50–30	90
	4	30–20	120
	5	20–10	45
	6	10–5	30
	7	5–2.5	45
	8	2.5–0	45
2	1	400–200	120
	2	200–100	150
	3	100–50	45
	4	50–30	60
	5	30–10	45
	6	10–5	45
	7	5–2.5	60
	8	2.5–0	45
3	1	900–500	240
	2	500–200	240
	3	200–100	75
	4	100–50	60
	5	50–20	75
	6	20–10	30
	7	10–5	60
	8	5–0	30

Samples collected at all three stations were isolated into 1 L bottles, paying attention to plastic contamination on the deck. Samples isolated in bottles were fixed using 3% formalin. Thus, the biological effect that may occur on plastics is prevented. The fixed samples were stored in black boxes in a cool shade to protect them from UV during the cruise.

After the samples were transported to the laboratory, they were stored in a cool and dark room. Then all collected samples were separated 10% of each sample using the Motoda splitter (Motoda, 1959). Four samples were selected from the prepared sub-samples for each station, as plastic identification analyzes have lengthy procedures. However, since the volume of

water filtered with MOCNESS during samplings was tens of m^3 (Table 2.2.1. shows how many m^3 of water were filtered in each isolated depth sample), it was possible that the analysis of the obtained plastic particles may take a long time. Therefore, each sample was again separated to represent 0.1 m^3 in the volume of filtered water. Table 2.2.2. presents the depths selected for plastic identification and the sub-samples volume prepared for analysis from these depth samples.

Table 2.2.2. Sub-samples volumes represented 0.1 m^3 volume of water sample of each isolated depth filtered with MOCNESS for plastic identification analysis.

Stn.	Net	Depth (m)	Sub-samples volume for plastic identification analyses (μL)
1	2	75–50	0.347
	3	50–30	0.240
	7	5–2.5	0.467
	8	2.5–0	0.507
2	3	100–50	0.458
	4	50–30	0.283
	6	10–5	0.196
	7	5–2.5	0.327
3	1	900–500	0.072
	4	100–50	0.247
	5	50–20	0.272
	6	20–10	0.667

Sample treatment

Before starting the analysis, all equipment used was washed with distilled water. Seawater was extracted from the sub-samples prepared for plastic identity analysis. Then the 30 mL 30% H_2O_2 and 25 mL 0.05 M Fe (II) solution was added to eliminate the organic material (Masura et al., 2015). In addition, samples were heated to be 60 °C for the chemical solutions can work more actively. This heating protocol continued for 14 d (Nakano et al., 2021). At

the end of this process, 200 mL NaI solutions were added to the samples, and adjusted the specific gravity to 1.5. Then, the remaining particles were filtered using a polytetrafluoroethylene (hereinafter PTFE) filter (filter is 25 mm Ø, pore is 1 µm Ø). Then, PTFE filters were dried at room temperature in a desiccator for 12 h to eliminate the liquid on the filter and the particles. This operation was performed for all samples shown in Table 2.2.2.

Analysis of microplastics

The infrared absorption spectrum of the particles on the PTFE filter was measured. This measurement was done using micro-Fourier Transform Infrared Spectroscopy (hereinafter µ-FTIR) (IRT-7200, JASCO Co. Ltd., Tokyo, Japan) equipped with a linear array infrared microscope. The magnification of the µ-FTIR microscope was set to 16 x. Thus, the pixel length of the acquired image was measured as 12.5 µm. The infrared absorption spectrum measurement was adjusted as wavelength 400–4000 cm^{-1}. One quarter of the filter was included in the analysis due to both the long analysis time and the high number of particles. Since even the analyzed part of the filter is wider than the focus area, the 4 mm × 4 mm (320 pixels × 320 pixels) part was imaged each time. Therefore, a quarter of the filter was completely imaged with average eleven different positions.

As mentioned in the chapter I, there are dozens of different types of plastics. However, some types are produced and used more. The present study focused on five types of plastics that are used extensively (Geyer et al., 2017). The plastic types focused on the study: polystyrene (PS), polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), and polyethylene terephthalate (PET) (GESAMP, 2019; Wang et al., 2021). The correlation coefficients between the spectra of all analyzed suspicious particles and the spectra of five standard plastics were calculated for each type in a specific wavelength range (the wavelength of the five focused plastic types were shown in the Table 2.2.3.). Contour images of the correlation coefficient of each measurement area of each polymer type of these five plastic types were created.

Table 2.2.3. The wavelength of the five focused plastic types

Plastic types	Wavelength 1 (cm^{-1})	Wavelength 2 (cm^{-1})
Polyvinyl chloride (PVC)	1350–1800	–
Polystyrene (PS)	1350–2250	–
Polyethylene terephthalate (PET)	2740–3300	–
Polyethylene (PE)	2740–3300	1350–2250
Polypropylene (PP)	2740–3300	1350–2250

First, to determine whether the particles on the filter are plastic, the particle was considered a plastic candidate if the suspect particle had a correlation coefficient exceeding a threshold calculated as the sum of the mean values and three times the standard deviation of the correlation coefficients. The spectra of the plastic candidate particles were compared using the commercial library KnowItAll (Bio-Rad Laboratories Inc., CA, USA). With this comparison, it was confirmed that the suspected particle belonged to a polymer type. The color image map of the correlation coefficients was converted to a bicolor figure to determine particle sizes using imaging software. Then, the dimensions of each plastic particle were measured (minor and major) using the ImageJ software (Schneider et al., 2012).

Calculation of microplastic volume

The plastics get smaller by crumbling with some effect, as mentioned in the previous sections. Particles formed during this crumbling occur regardless of basic geometrical shapes. Therefore, it would be inappropriate to calculate the volumes of the captured μPs particles directly from their measurements. Although 3D measurements are better to learn the volume of a particle, it gives 95% accurate results only in major and minor dimensions (Bagheri et al., 2015). In addition to the minor and major dimensions obtained via ImageJ, angle (The angle between the primary axis and a line parallel to the *x*-axis of the μPs particle image), and area (surface area of the μPs particle found by converting from square pixels measured from the image), which are related to these the two data (minor and major), were also obtained. Thus, the area of each μPs particle was applied to the formula "equivalent spherical diameter" (hereinafter ESD), and its approximate spherical volume was determined (Colas et al., 2018). The ESD formula defined as

$$ESD = 2 \times \sqrt{\left(\frac{Area}{\pi}\right)}$$

(2.1)

where ESD is equivalent spherical diameter and *area* is area of µPs particle. After obtaining the ESD of each particle, its volumes were calculated. The sphere volume formula defined as

$$V_{\text{sphere}} = \frac{4}{3}\pi r^3$$

(2.2)

where V_{sphere} is volume of sphere and *r* is radius. After calculating the volume of each particle, the total surface area of the plastics at each sampling depth was calculated. The total volume of µPs particles formula expressed as

$$\sum_{i=1}^{n} \mu PsV_1 = \mu PsV_1 + \mu PsV_2 + \mu PsV_3 + \mu PsV_4 + \cdots + \mu PsV_n$$

(2.3)

where μPsV is volume microplastic particle.

Calculation of microplastic surface area

The surface area of each µPs particle was calculated with the diameter measure obtained from the ESD formula (2.1) used for volume calculation. The sphere surface area formula defined as

$$A_{\text{sphere}} = \pi r^2$$

(2.4)

where A_{sphere} is surface area of sphere and *r* is radius. After calculating the surface area of each µPs particle, the total surface area of the µPs at each sampling depth was calculated. The total surface area of µPs particles formula expressed as

$$\sum_{i=1}^{n} \mu PsA_1 = \mu PsA_1 + \mu PsA_2 + \mu PsA_3 + \mu PsA_4 + \cdots + \mu PsA_n$$

(2.5)

where μPsA is surface area microplastic particle.

Results

A total of 1875 m^3 of the water column was filtered at Stn. 1, Stn. 2, and Stn. 3, respectively, 495, 570, and 810 m^3, with eight nets other than the first dive net (the stations and filtered volumes of sampled depths are shown in Table 2.2.1.). As stated before, four different depths were analyzed from each station (the analyzed sample depths and analyzed quantities are shown in Table 2.2.2.). As a result of the µ-FTIR analysis, plastic materials were found at all depths and stations. The plastic particles found vary in size from 30 µm to 345 µm. However, since the mesh size used is 64 µm, only µPs particles larger than the mesh size were considered in the present chapter. Thus, the plastic numbers determined per station in the analysis of the sub-samples were as follows; 83 in Stn. 1, 60 in Stn. 2, 35 in Stn. 3 (in total 178). The distribution of the depths by stations and the proportional plastic number per 1 m^3 were shown in the Table 2.3.1.

Table 2.3.1. Distribution of the microplastic numbers depending on the depths in the stations.

Stn.	Net	Depth (m)	1 m^3
1	2	75–50	280
	3	50–30	1120
	7	5–2.5	1480
	8	2.5–0	440
2	3	100–50	680
	4	50–30	640
	6	10–5	680
	7	5–2.5	400
3	1	900–500	280
	4	100–50	600
	5	50–20	280
	6	20–10	240

Composition of microplastic types

Different plastic-type compositions were obtained at each station and depth during the μ-FTIR analysis. The most common type in the composition of the identified μPs was determined as PE (21%). The other frequently encountered μPs types were determined as PP (15%) and PET (15%). The remaining 49% were classified and calculated as the "other" group (this group included copolymers such as PE/PP, PP/PE, PE/Acryl, and other types of plastic polymers). Percentage distribution was also calculated for the stations. (i) The densest type of μPs detected at Stn. 1 was PE (18%), followed by PP (12%) and PET (4%). The remaining 66% were in the other group. (ii) The densest type of μPs detected at Stn. 2 was PE (26%), followed by PET (25%) and PP (17%). (iii) The remaining 32% were in the other group. The densest type of μPs detected at Stn. 3 was PET (23%), followed by PE (20%) and PP (17%). The remaining 40% were in the other group. The percentages of μPs numbers and types depending to the depths as well as the stations are shown in Figures 2.3.1, 2.3.2, and 2.3.3.

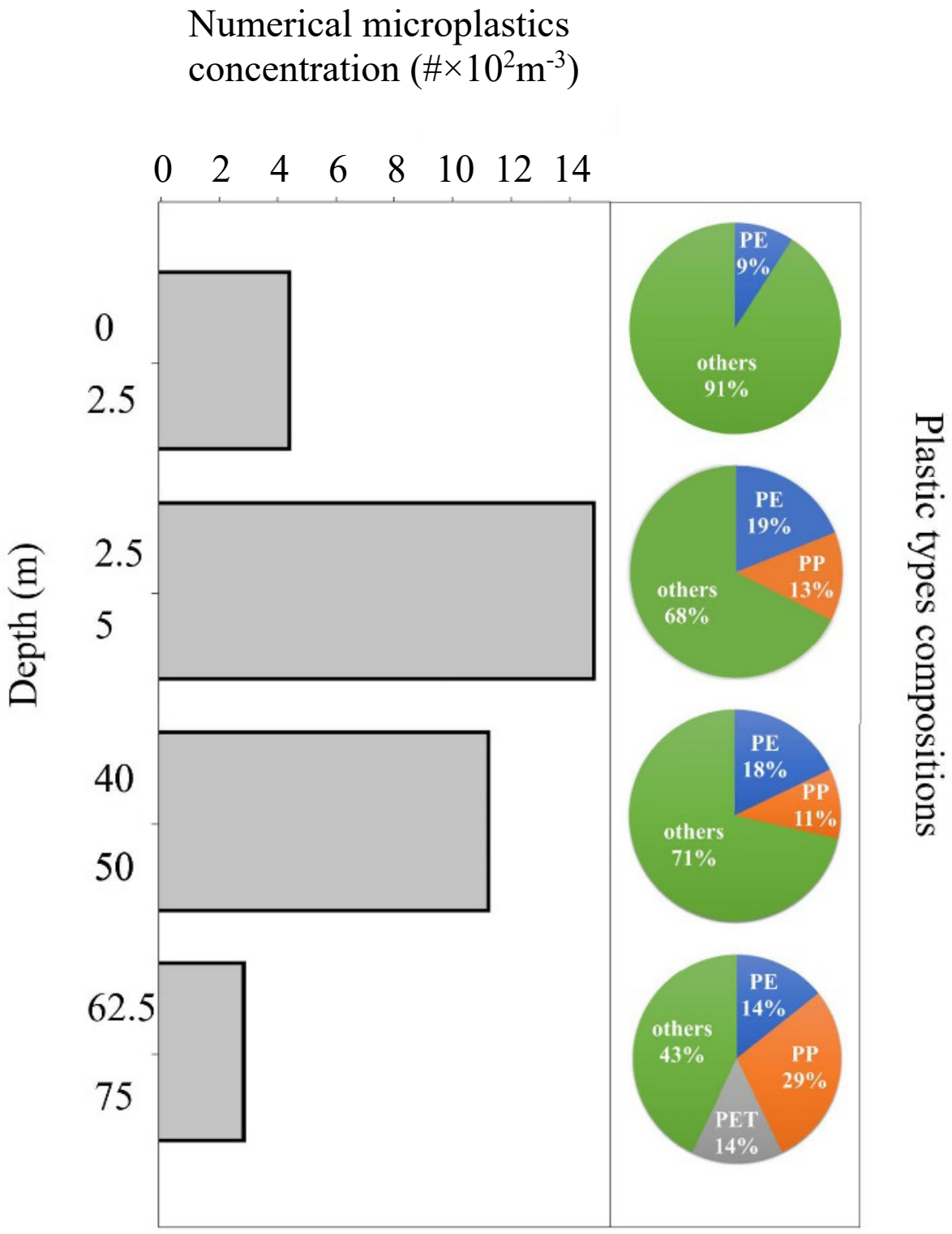


Figure 2.3.1. Depth-dependent microplastic type compositions and particle numbers in Stn. 1.

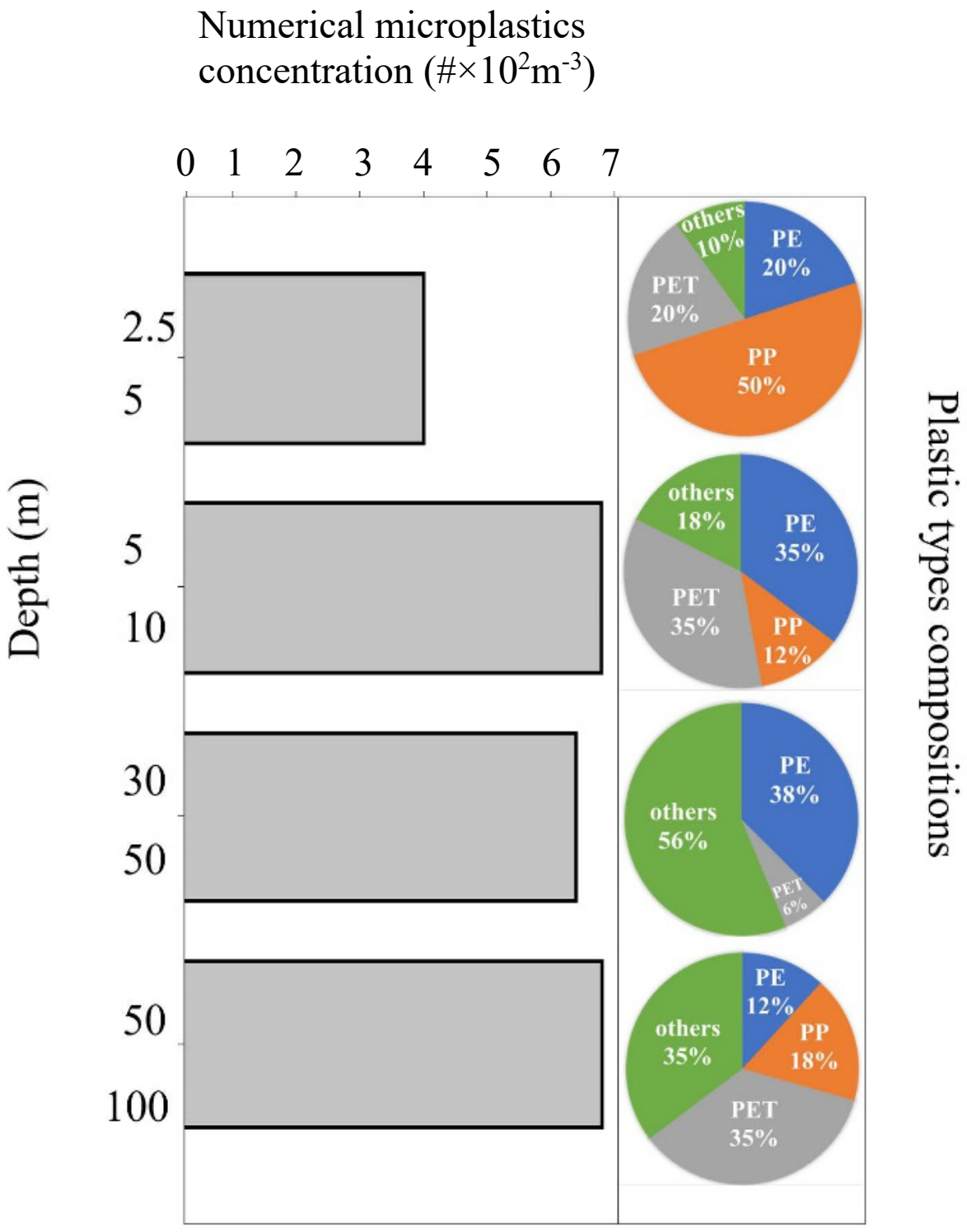


Figure 2.3.2. Depth-dependent microplastic type compositions and particle numbers in Stn. 2.

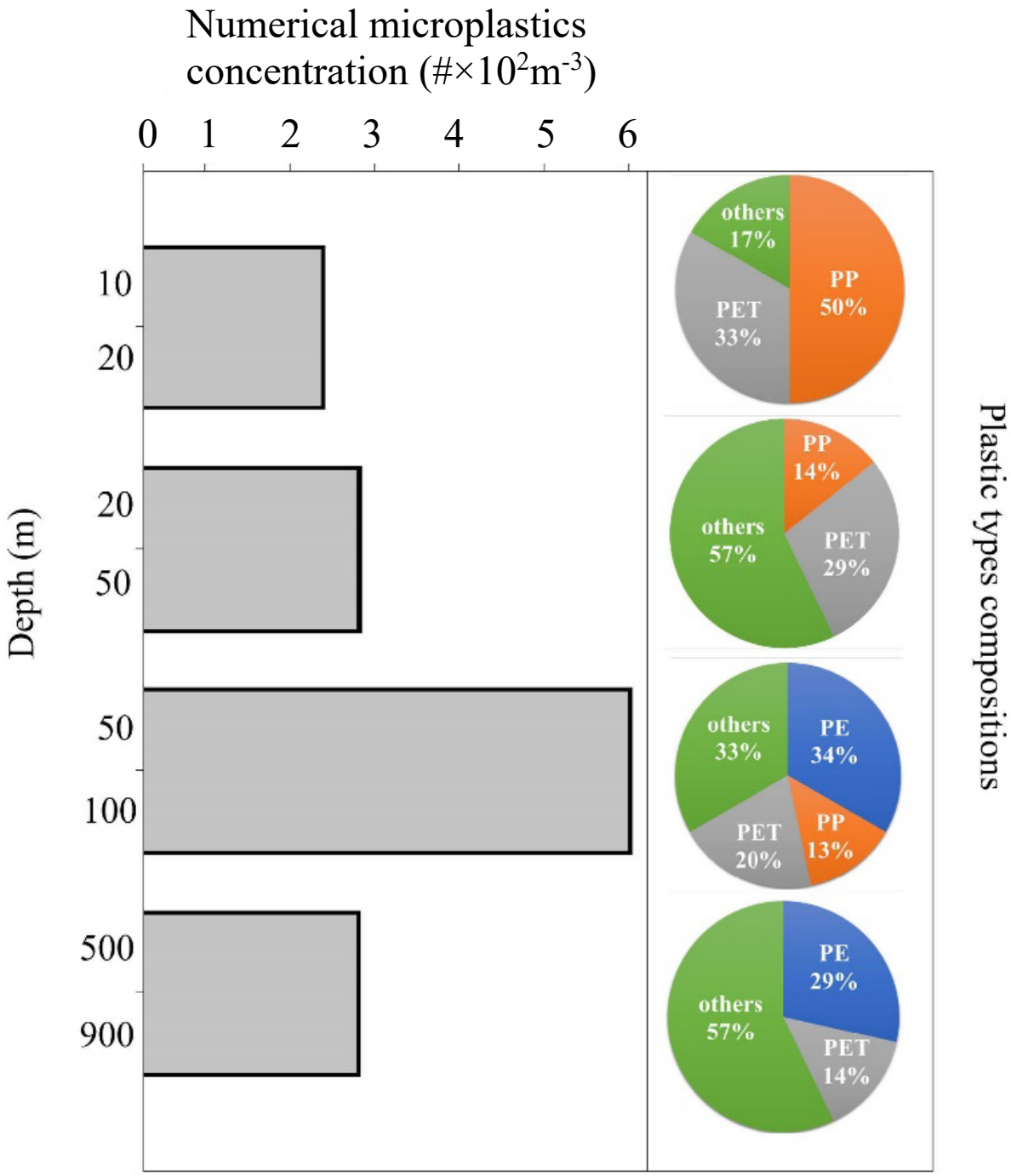


Figure 2.3.3. Depth-dependent microplastic type compositions and particle numbers in Stn. 3.

Size distribution of microplastics

The sizes of the µPs particles obtained from the sampling field and analyzed for types of identities differed by the stations and the depths. Plastic sizes were grouped into five different classifications as 64–100 µm, 101–150 µm, 151–200 µm, 201–300 µm, and 301 µm–above. The maximum number of plastic particles was classified in the 64-100 m range group (60%), in all three stations. The percentages of the other groups were determined as 101–150 µm (19%), 151–200 µm (11%), 201–300 µm (8%), and 301 µm–above (2%), respectively. (i) The ordering of these size distribution groups for Stn. 1 was determined as 64–100 µm (57%), 101–150 µm (22%), 151–200 µm (12%), 201–300 µm (7%), and 301 µm–above (2%). (ii) The ordering of these size distribution groups for Stn. 2 was determined as 64–100 µm (63%), 101–150 µm (17%), 151–200 µm (11%), 201–300 µm (7%), and 301 µm–above (2%). (iii) The ordering of these size distribution groups for Stn. 3 was determined as 64–100 µm (66%), 101–150 µm (17%), 201–300 µm (11%), 151–200 µm (7%), and 301 µm–above (0%). The size distribution of the depth samples is shown in the Figures 2.3.4, 2.3.5, and 2.3.6.

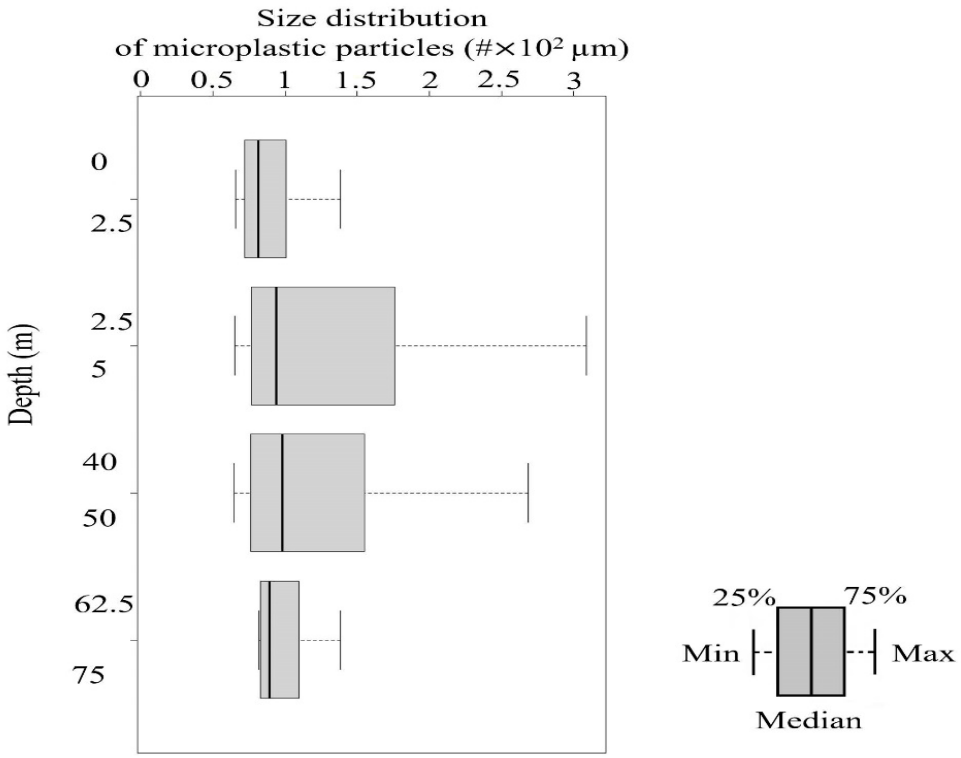


Figure 2.3.4. Size distribution of microplastics in Stn. 1.

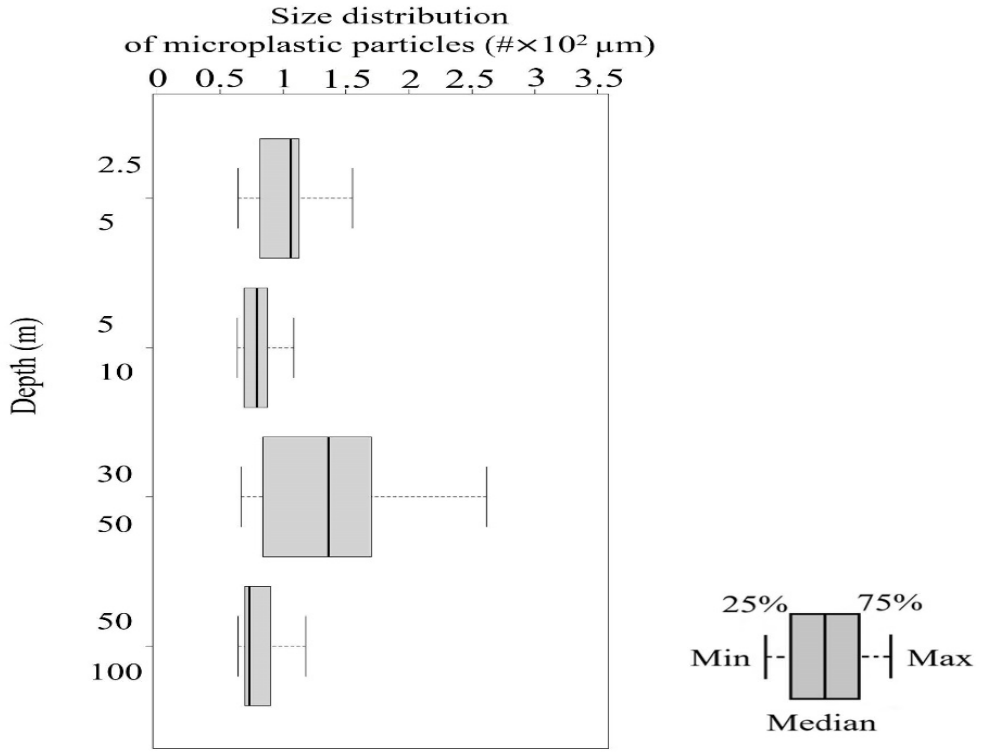


Figure 2.3.5. Size distribution of microplastics in Stn. 2.

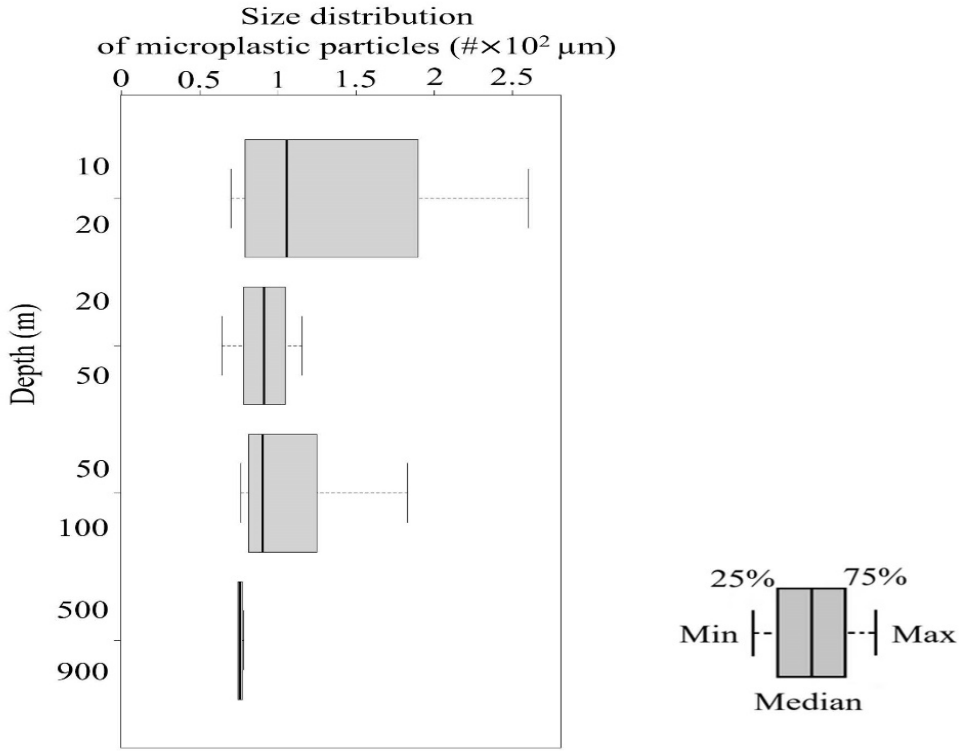


Figure 2.3.6. Size distribution of microplastics in Stn. 3.

Microplastic volume

The total volumes of μPs obtained from the samples of each isolated depth and station were calculated. Since the analyzed sub-samples represented a volume of 25 L of each sampling depth, the concentration to 1 m^3 was calculated. Then the measure in μm^3 was converted to mm^3 and processed. The total volumes of microplastics were found to be 4.07 mm^3 for a 1 m^3 water at each sampling depth. (i) The total μPs volume was found to be 1.671 mm^3 in Stn. 1. (ii) The total volume of μPs was found to be 1.631 mm^3 in Stn. 2. (iii) The total volume of μPs was determined as 0.768 mm^3 in Stn. 3. The μPs volumes of each depth and station were shown in the Table 2.3.2.

Table 2.3.2. Volume of the microplastics depending on the depths in three stations.

Stn.	Net	Depth (m)	$mm^3 \cdot m^{-3}$
1	2	75–50	0.069
	3	50–30	0.558
	7	5–2.5	0.838
	8	2.5–0	0.206
2	3	100–50	0.184
	4	50–30	0.285
	6	10–5	0.966
	7	5–2.5	0.196
3	1	900–500	0.025
	4	100–50	0.466
	5	50–20	0.074
	6	20–10	0.203

Microplastic surface area

In addition to the volume calculations, the surface areas of the detected plastics were calculated using the ESD formula. Also, since the rate represented by the sub-sampling was 25 L, the ratio to 1 m^3 was calculated. Then the measure in μm^2 was converted to mm^2 and processed. The total surface area of µPs were found to be 165.557 mm^2 for a 1 m^3 water column in each sampling depth. (i) The total µPs surface area was found to be 83.283 mm^2 in Stn. 1. (ii) The total surface area of µPs was found to be 46.468 mm^2 in Stn. 2. (iii) The total surface area of µPs was determined as 35.806 mm^2 in Stn. 3. The µPs surface area of each depth and station were shown in the Table 2.3.3.

Table 2.3.3. Surface area of the microplastics depending on the depths in three stations.

Stn.	Net	Depth (m)	$mm^2 \cdot m^{-3}$
1	2	75–50	4.824
	3	50–30	27.278
	7	5–2.5	40.387
	8	2.5–0	10.794
2	3	100–50	11.789
	4	50–30	16.167
	6	10–5	8.336
	7	5–2.5	10.176
3	1	900–500	2.736
	4	100–50	19.549
	5	50–20	4.988
	6	20–10	8.533

Discussion

The present chapter focused on the earth's largest living space, the water column, rather than the surface waters that most plastic pollution studies have focused on to date. Another point that differed from the general was the mesh size. Although in mainly microplastic studies, samples are collected with 300 µm mesh size nets, in the present study, 64 µm mesh-size nets were used to capture smaller particles. Thus, a more realistic idea of plastic pollution in the water column was provided. As a result of the analyzed sub-samples, only three µPs particles larger than 300 µm were found, which makes up 2% of the total found µPs particles. The most abundant size group of the plastics found was 64–100 µm (60% of total µPs volume). Considering the other size groups (64–100 µm (60%), 101–150 µm (19%), 151–200 µm (11%), 201–300 µm (8%), 301 µm–above), the number of plastics increases as the size of the plastics decreases. The individual situations of the stations were the same as the general. The ratios of plastic particles in the size range of 64–100 µm to total particles; 57% in Stn. 1, 63% in Stn. 2, and 66% in Stn. 3. The increase in the number of particles as the particle size decreases in water column plastic pollution is not a new finding. A similar finding was demonstrated by Egger et al. (2020) in a study on the North Pacific Garbage Patch. In this study, they sampled with 333 µm nets settled MOCNESS and focused on particles between 500 µm and 5 mm. The findings of their study showed that 52% of the plastic particles they detected were smaller than 1.5 mm. It is also understandable that there is a threshold of 1.5 mm. For this case, Cózar et al. (2014) showed that the number of plastics up to 2 mm increased as their size decreased, but the number of plastics less than 1 mm in size decreased with decreasing size. However, the difference and the important point in the present study is to catch the particles that cannot be caught with the 300 µm mesh net used chiefly. Thus, it reveals the status of plastic pollution under 300 µm. As a result of sub-sample analysis, only 3 of the 178 plastic detected particles (lowest limit is 64 µm) were above 300 µm. This position shows that the smallest possible opening should be used for studies to determine the water column's plastic pollution volume. In the present study, 64 µm, which is the smallest mesh opening for MOCNESS (Wiebe et al., 1985), was used. However, the size groups show that there is a 41% difference in percentage between 64–100 µm (60% of total) and 101-150 µm (19% of total), which raises doubts that more plastic can be found under 64 µm. This may suggest that the issue of plastic pollution is getting bigger as we look at its small size for detection. Thus,

the missing puzzle pieces of the plastic called “missing plastic” (Cózar et al., 2014) can be completed more easily.

Another issue related to plastic sizes was the differences between sampling depths. Although it showed a similar size distribution between the surface and 100 m depth, it showed a very narrow range at 500–900 m depth. For example, Figure 2.3.4. shows the distribution of various depths from the surface to a depth of 75 m. Here, although it is seen in particles above 300 µm, it is clearly seen that the widespread distribution was between 64 µm and 150 µm. Likewise, the situation is similar for Stn. 2, shown in Figure 2.3.5. However, as shown in Figure 2.3.6, the situation in the 500–900 m depth range differed from other stations and depths. The size range of the particles found in this depth range was between 67 µm and 77 µm. The sinking behavior of the particles can support this situation according to their sizes, which Kooi et al. (2017) explained using the theoretical model. Their study showed that for plastic particles smaller than 10 µm in size continued to sink irreversibly after they began to sink. Their work showed that plastic particles smaller than 10 m in size, they continued to sink irreversibly after they began to sink. However, they revealed that the 1 mm to 10 mm particles also sink, but resurface by oscillating like a yo-yo, and then the particles repeated this process. As they stated in their studies, particles with a size of 1 mm or larger have a much slower biofouling settling than those with a size of 10 µm. They also stated that biofouling may be more effective than the size or plastic density of particles sinking. With occurrence of biofouling or marine snow, particulate organic and inorganic materials can be included in a complex structure. Thus, the densities of the plastics themselves become insignificant in the structure. Therefore, different plastic sizes have different fates in the ocean water column. From their perspectives, a narrow size range of 500–900 m can explain the observed particle sizes in the present study.

In addition, another important point about the results is the distribution of the detected plastic types, the PE type was the densest at 21%, out of all stations of the five plastic types (PE, PP, PET, PVC, and PS) focused during the µ-FTIR analysis. PP (15%) and PET (15%) types with the same ratios shared the second place. Although PE took the first place in terms of all stations, PP (second place in Stn. 1) and PET (second place in Stn. 2 and 3) alternately shared the second place. The distribution of plastic types according to depths and stations were shown in Figure 2.3.1. for Stn. 1, 2.3.2 for Stn. 2, and 3. As expected, a higher specific gravity than seawater plastic type such as PET was found at all depths. In additions, it was observed that floating plastic types (lower specific gravity than seawater) such as PE and PP

were distributed at all the depths. Although it is possible for these floating particles to sink by physical effects, this is not considered very revealing (Kooi et al., 2017b). It has been repeatedly suggested by some studies that the biofouling effect is the biggest role in the sinking of particles (Long et al., 2015; Möhlenkamp et al., 2018; Kvale et al., 2020). The role of biofouling and the resulting marine snow structure in the vertical travel of microplastics is discussed further in the Chapter III.

In the Chapter I, it was explained extensively that plastics do not disappear in nature, but only get smaller with some effects and how this effect occurs. Thanks to the crumbling process, the chance of plastics entering the food web increases. In order to explain it more efficiently, it is more logical to use the food pyramid, which is the hierarchical structure of the food web. The number and biomass of living creature increase with every step taken from the top to the bottom in the food pyramid structure (Campbell et al., 1999). For this reason, it would not be wrong to say that increasingly crumbling plastics will be easier to enter the ecosystem. In addition, the crumbling of a plastic entering the system causes a particle to turn into hundreds of smaller particles. This issue can be explained from the surface areas and volumes of the plastic particles obtained in the present study. As a result of the plastic analysis of the samples collected from all stations and depths, the total volume of the detected particles was 4.07 mm^3 in 12 m^3 (the Table 2.3.2. shows the volumes of the plastic particles from the depths and stations). To visualize this value indicated, the volume of the 1-yen coin (diameter is 20 mm, thickness is 1.5 mm) is approximately 675.44 mm^3. Also, the total surface area of the same particles was 165.55 mm^2 in 12 m^3 (the Table 2.3.3. shows the surface areas of the plastic particles from the depths and stations). To visualize this value, the surface area of 1-yen is 235.62 mm^2. It shows that although the number of plastic particles obtained from subsamples was high, their volume and surface area are quite small. Based on the data of the present study, an object with a surface area of only 70% of 1-yen can crumble into 7120 pieces (the total number of particles detected, the particle distribution of stations and depths are given in the Table 2.3.1.) and disperse into the system. Although 1-yen is too large to be eaten by zooplankter, it can become edible by thousands of zooplankton, via to the breakdown and crumbling of the plastic. The relationship of zooplankton and microplastics was discussed extensively in the Chapters IV and V.

Moreover, to examine the estimated sources of plastic pollution in the region, the characteristics of the field of study are as follows. Although the data from three stations is not enough to get an idea about a sea, the concept is based on the literature knowledge of the region. The

present study was realized in the Sea of Japan, which is called the miniature ocean (Ichiye, 1984). This sea, which has almost every feature of an ocean, is surrounded by Japan, Russia, North, and South Korea. All the surrounding countries are industrialized and use the resources and coasts of this sea both economically and as a anthropologic living space (Danchenkov et al., 2006). Although Russia is the most populated country among the neighboring countries, the population density of this country is mostly concentrated in the western part (World Population Review, 2022b). The population density of Japan, which is the next most populous country, is located on the coast of the Pacific Ocean, with a rate of 81% of the total population (Statistics Bureau Japan, 2021). North and South Korea are in the form of peninsulas (surrounded by the Sea of Japan and the Yellow Sea); their most populated cities are on the seacoast (World Population Review, 2022a, World Population Review, 2022c). However, the Sea of Japan is not only under the influence of neighboring countries in terms of plastic pollution. The Tsushima Strait, located between Japan and South Korea (33.27°N, 130.25°E) in the region's south, connects to the East China Sea. This strait feeds the Sea of Japan from the Kuroshio Current, the world's one of major plastic-carrying current (Nakajima et al., 2021). It is also under the influence of the Taiwan Warm Current (Isobe, 1999) passing over the East China Sea, fed by the Yangtze River, which discharges the largest plastic load in the world into the ocean (Lebreton et al., 2017). So, it would not be wrong to say that these two warm currents reach the Sea of Japan (Tsushima Warm Current) and the plastic load. Moreover, Iwasaki et al. (2017) showed that the journey of the plastic via Tsushima Current into the Sea of Japan system was dragged and accumulated, especially on the shores of Japan, with the stokes drift effect. This makes the coast of Japan the unluckiest part of the region in terms of plastic pollution.

CHAPTER III: Microplastic particles smaller than 64 µm. Usual suspect: Marine snow

Preface

The present chapter, unlike other chapters, was not targeted. It was born with the support of the analysis results of the Chapter II of the doubt that arose during the analyzes of the Chapter IV. This section was created to explain the appearance of plastic parts smaller than the size of the 64 µm mesh used in sampling in the FTIR results. In Chapter II, the fact that the positive floating plastics of the water column has decreased to 900 m deep in this chapter shows that a carrier should be a carrier. For this reason, with the help of microscope images, the hypothesis that this transport took place by structures such as marine snow and aggregate was discussed in this section.

Introduction

Understanding the ability of plastic to act in the aquatic system is important to learn the extent of its pollution. It was mentioned in the previous sections about the transport of plastic material away from the pollutant sources by the movements of water in the aquatic system. The dispersion movements of plastics on the ocean surface are a relatively well-known issue (Cózar et al., 2014; Ruiz et al., 2020; van Sebille et al., 2020). In addition, plastic types with both negative and positive buoyancy have become part of the sediment (Van Cauwenberghe et al., 2013; Nakajima et al., 2021). The water column, which is the region between the surface and the sediment, is a relatively less known area for plastic pollution. There are both physical and biological parameters that are known to be effective in the transportation of positive buoyancy plastics to the sediment. Physical forces affecting the vertical transport of plastic particles are such as Stokes drift, Lagrangian and Eulerian flow, Langmuir circulation, ice melting, suspended inorganic material, hyperpycnal flow, and river plumes (van Sebille et al., 2020). However, as mentioned in the previous chapter, the effect of physical transport is relatively weak. The more significant impact is thought to be biological activities (Kooi et al., 2017a). For instance, the plastic particles that are absorbed by living creatures in vertical movements while feeding in surface waters can leave as fecal pellets in deep waters after vertical migration (Choy et al., 2019). Likewise, the fecal pellet can be given as an example for

vertical transport, since it is also deposited directly into the system from the surface (Cole et al., 2016). Another biological reason is biofouling (Kooi et al., 2017a). Biofouling organisms, also called epibiont organisms (bacteria, algae, sessile organisms), when they come across a surface, settle on the object (Lewis, 1998). These living things can change the specific gravity of the object on which they are placed and change the object's negative buoyant, even if it is positively buoyant (Ryan, 2015b). The ethylene-butyl acrylate (EBAK) plastic bottle collected on the rocky breakwater zone of Odaiba beach (35.62°N, 139.77°E) showed in the Figure 3.1.1. to demonstrate the settlement of biofouling organisms on the material.

Figure 3.1.1. Biofouling organisms such as algae, mussels and barnacles that have grown on an ethylene-butyl acrylate plastic bottle. The left side of the Figure shows the situation in the field where the bottle is located, and on the right side, it is shown together with the scale in the laboratory. In addition, it showed one side of the bottle in two photographs and conveyed the biofouling organisms on it.

Also, the photograph of a part of the bottle taken with a digital microscope is presented in the Figure 3.1.2. This Figure was also supported by the 3D image while showing the algal bio-fouling layer developing on the plastic material.

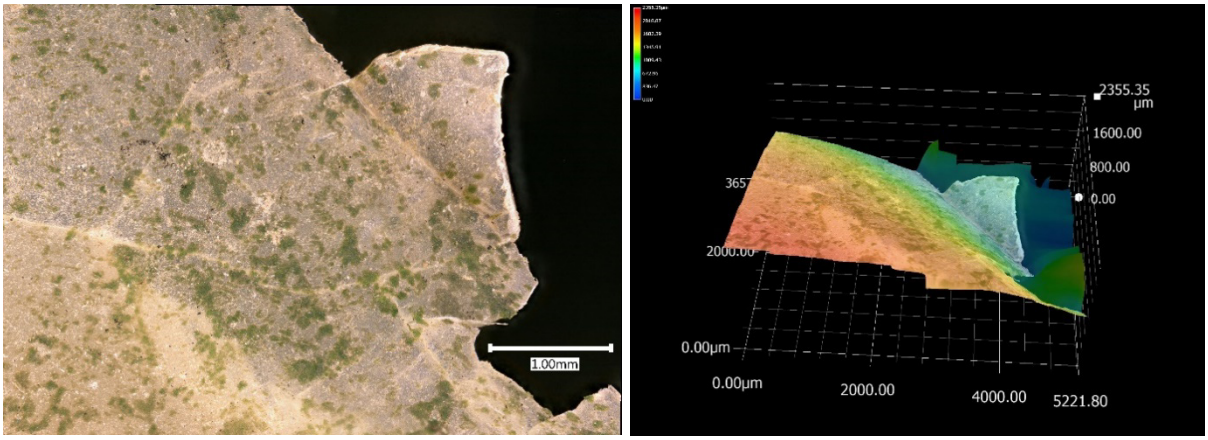


Figure 3.1.2. The photograph of a part of the bottle taken with a digital microscope and 3D visual support.

Another vertical plastic carrier biological structure is marine snow. Marine snow is the major structure of the biological pump system. The structure is the macroscopic (>500 µm) structure formed by organic aggregates (Turner, 2015). Biofilm organisms developing on plastic materials (Rummel et al., 2017) may affect the plastic's buoyancy over time, as seen in the Figure 3.1.2. Marine snow formation may occur due to the contamination of the algal structure that has already formed with detritus structures from the environment. Thus, while the plastics can be the beginning of the marine snow, they can also be included in the structure by the adhesion of the plastics to the detritus structure. Thus, plastics travel vertically in a structure independent of their specific gravity (van Sebille et al., 2020).

It was stated in the previous section that the Sea of Japan sampling was carried out with MOCNESS equipped with 64 µm nets. However, plastic particles smaller than 64 µm were detected as a result of µ-FTIR analysis. Although particles smaller than the mesh opening (64 µm) cannot be included in the issue of plastic distribution, they cannot be ignored either. Therefore, 64 µm was determined as the threshold, and the larger ones were interpreted for distribution in the Chapter II, while the smaller ones were interpreted in the present chapter. In addition, the hypothesis was supported with the particles of various colors and sizes, which were noticed in the possible marine snow (also aggregates) structures encountered during the

microscope analysis of the Chapter IV. The present chapter aims to interpret the results with (i) the principles of the ocean system's operating system and (ii) possible ideas about the vertical journey of positive buoyant plastic.

Materials and methods

The present chapter was born with the Chapter II and IV results. Therefore, it is the same as some parts of the material method sections of the other chapters mentioned. The study area, sample collection and preparation, sample treatment, and analysis of microplastics sections of the material method section of Chapter II are valid in the present chapter. The fact that the same digital microscope (Keyence VHX6000, Keyence Corporation, Osaka, Japan) was used with the same technique is the same as Chapter IV. The present section is given this way to avoid repetition in the thesis text.

Results

The smallest particles identified as plastic were determined to be 30 μm due to μ-FTIR analysis. The total number of 325 plastic particles (the analyzed volume for each depth range is 25 L) was detected smaller than 64 μm, which was determined as the upper limit. However, the total number of plastics reached 13×10^3 at the ratio of the number of particles determined for the volume of 25 L to 1 m^3. The number of plastics per station was calculated as 4680, 4400, and 3920, respectively. The plastic numbers and type variations depending on the depth of each station are shown in Tables 3.3.1. (Stn. 1), 3.3.2. (Stn. 2), and 3.3.3. (Stn. 3), respectively. Since the focused particles are smaller than the mesh opening, not all particles can be captured in this size range. Therefore, it was shown with the variation instead of using the distribution.

Table 3.3.1. Depth-dependent plastic concentrations and type variations of Stn. 1.

Depth (m)	Concentration (# m^{-3})	Type variation
62.5–75	40	50%PP, 50%others
40–50	1040	23%PP, 19% PE, 12% PET, 4% PVC, 42% others
2.5–5	2680	16% PP, 2% PE, 1% PVC, 81% others
0–2.5	920	22% PET, 17% PE, 9% PP, 52% others

Table 3.3.2. Depth-dependent plastic numbers and type variations of Stn. 2.

Depth (m)	Number of plastics (# m^{-3})	Type variation
50–100	800	25% PET, 15% PE, 10% PP, 50% others
30–50	1320	49% PE, 9% PP, 42% others
5–10	1640	39% PE, 10% PP, 10% PET, 41% others
2.5–5	640	37% PE, 13% PP, 50% others

Table 3.3.3. Depth-dependent plastic numbers and type variations of Stn. 3.

Depth (m)	Number of plastics (# m^{-3})	Type variation
500–900	1160	55% PE, 10% PP, 4% PET, 31% others
50–100	680	18% PET, 18% PP, 17% PE, 47% others
20–50	840	24% PP, 14% PE, 14% PET, 48% others
10–20	1240	29% PET, 16% PP, 10% PE, 45% others

Particles of suspected plastic in various sizes, shapes, and colors were noticed in the aggregates structures that were noticed during the microscope analyses. In addition, similar particles were observed in different densities in the aggregates and marine snow structures at all three station's 24 depth layers. The particles included in this marine snow and aggregate structure were shown in Figure 3.3.1. from the Stn. 3, which was sampled deeper than the other stations.

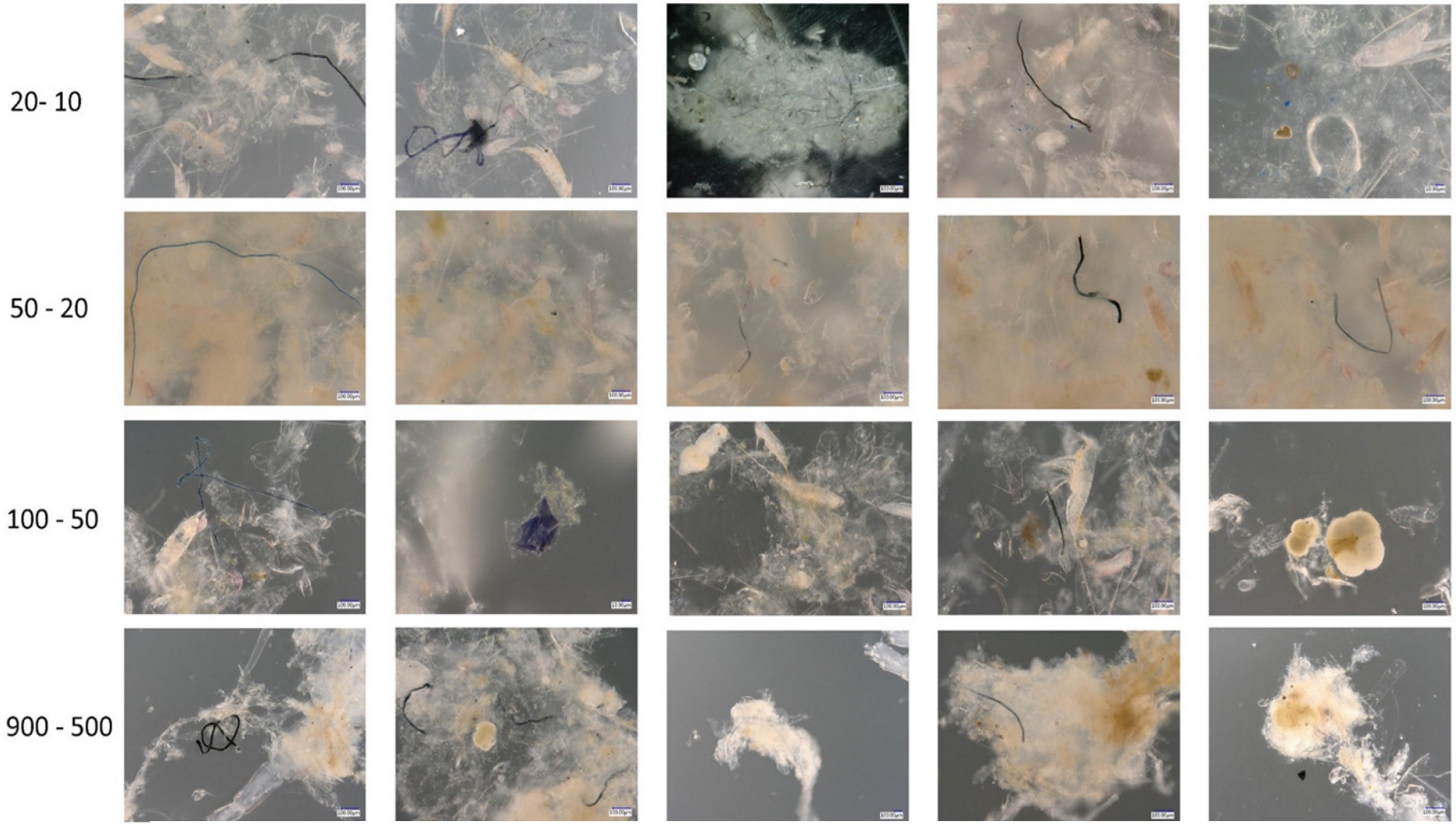


Figure 3.3.1. In the samples collected from vertical sampling in the Sea of Japan, there are suspicious plastic-like particles of various colors and shapes in marine snow structures. The sampling depths are shown in m in the left column.

Discussion

In the present chapter, the potential effect of marine snow on the vertical journey of positive buoyant plastics was hypothetically explained with supporting clues. Since the specific gravity of positive buoyant plastics is lower than that of water, it was theoretically expected that they would not sink. However, the results of Chapter II and Chapter III show that the predominant presence of positive buoyant plastics such as PE and PP can be seen. In addition, the other situation that was not expected to happen theoretically is the number of particles smaller than 64 µm from the water column filtered by MOCNESS nets with 64 µm mesh and the diversity of types in their variances. To clarify this unexpected situation, it can be interpreted in terms of members of the vertical transport of the aquatic system (Alldredge and Silver, 1988; Turner, 2015). Marine snow and aggregates structures, which are among the aforementioned carrier members for the present study particle size range, stand out among others (Kooi et al., 2017a). Several studies show that substances with a specific gravity lower than water are vertically transported in the water column by marine snow and similar structures. For example, marine snow was found to transport surface oil into the sediment effectively in the Gulf of Mexico oil spill in 2010 (Daly et al., 2016). It would not be wrong to state that these structures have an effect on the vertical migration of plastics with positive buoyancy. In fact, it has been proven both *in situ* (Zhao et al., 2018) and theoretically (Kooi et al., 2017a) that the organic detritus (marine snow and aggregates) structures mentioned carry plastics vertically.

In the present study, instead of determining the amount of plastic transported by marine snow or aggregate, it is hypothesized that this structure is effective in plastic transport. Because even though all marine snow structures captured, it is possible that some of them escaped from the structure during the sampling and remained out of the sample. Therefore, the amount of plastic and types of diversity shown in Tables 3.3.1, 3.3.2, and 3.3.3. are not only negligible as contamination but to convey that they differ from each other. Conversely, in case of contamination on the deck or in processes behind it, similarity in types or numbers should be expected. However, the amount and type ranges shown are only to indicate that particles smaller than 64 µm were found.

The hypothesis in the capture of the particles is that the organic detritus larger than the mesh aperture and smaller particles accompanying it were trapped in the sample. This idea was supported by the demonstration of particles that appear to be plastic of different sizes and

shapes in the marine snow structure detected in the samples shown in Figure 3.3.1. In addition, a schematic representation showing this organic detritus structure together with a plankton net was given in Figure 3.4.1.

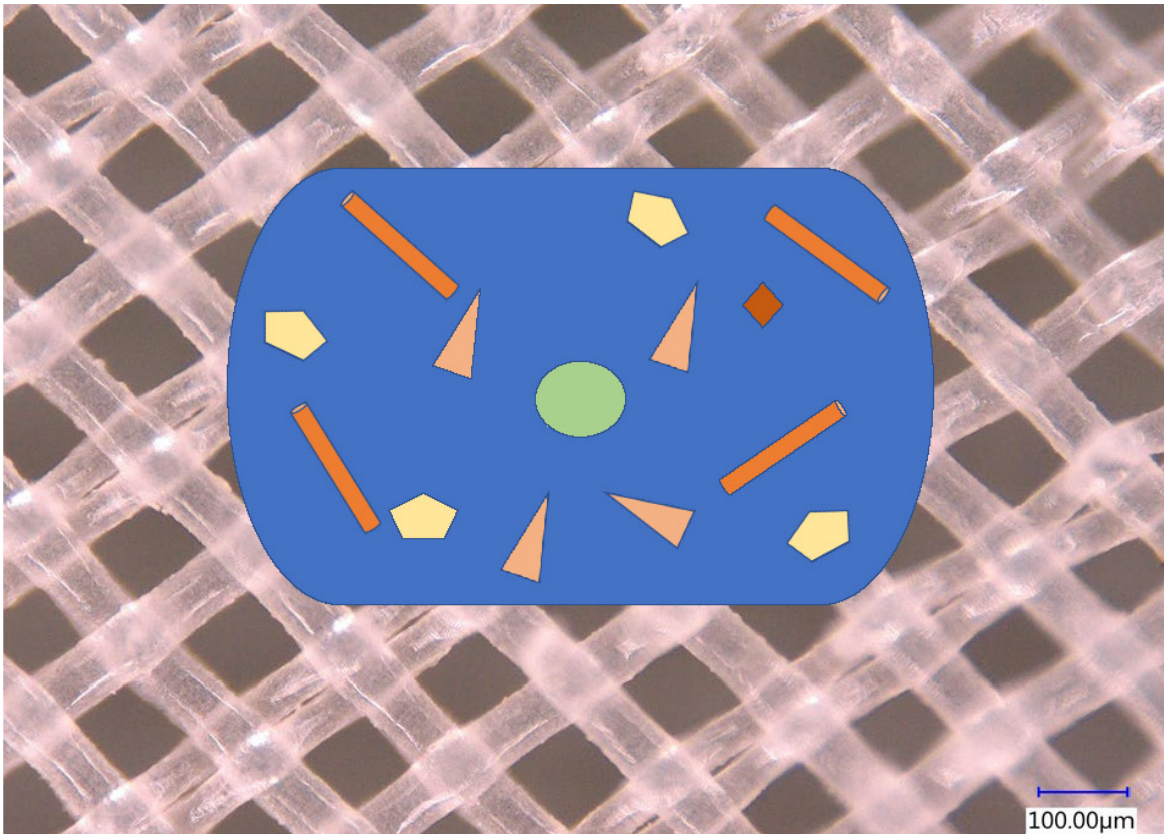


Figure 3.4.1. Schematic representation of the marine snow structure, and the particles smaller than the mesh opening carried by it. The net behind the structure is a 100 µm plankton net. Thus, the capture of the particle was visualized.

In addition, photographs supporting this hypothesis were shown on the following three pages (Figure 3.4.2.). These groups of photographs showed that some particles are smaller than the mesh size and that these parts are part of the marine snow structure. Therefore, the presence of Ps smaller than the mesh size in the sample, which was also reflected in the FTIR results, formed the hypothesis that these particles from the various colors and shapes in the photographs were transported to the aggregates or in the marine now structure.

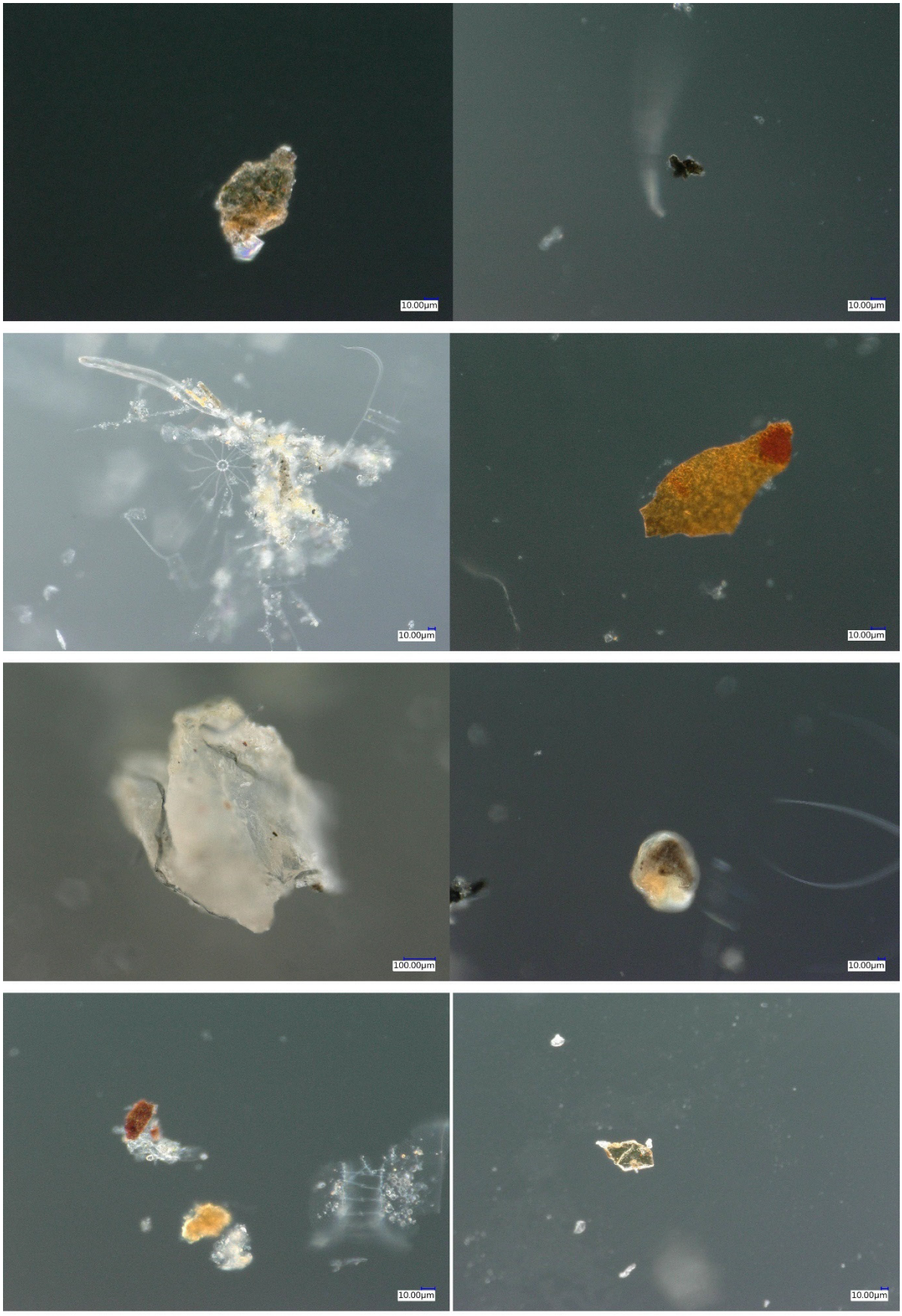


Figure 3.4.2. continued

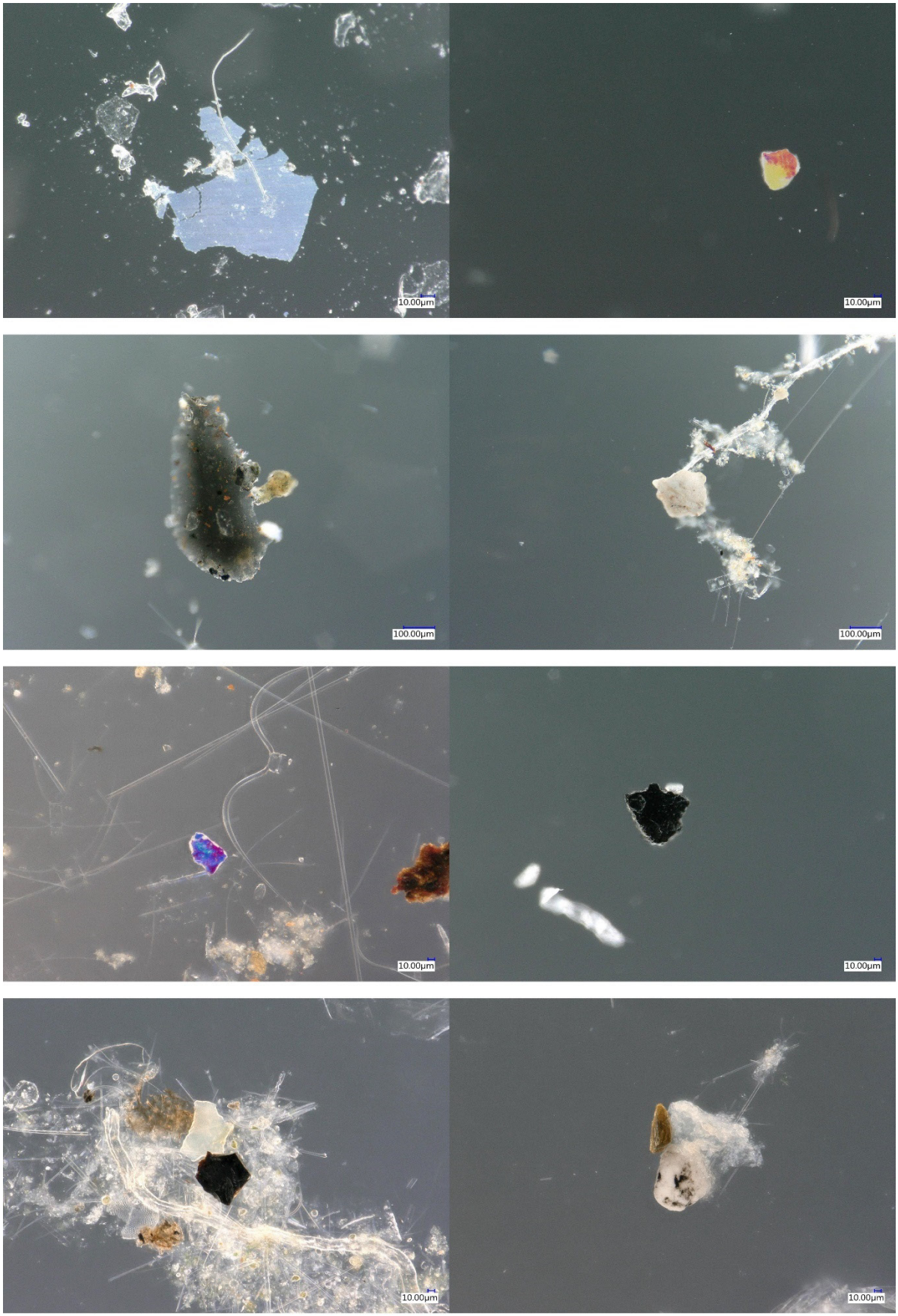


Figure 3.4.2. continued

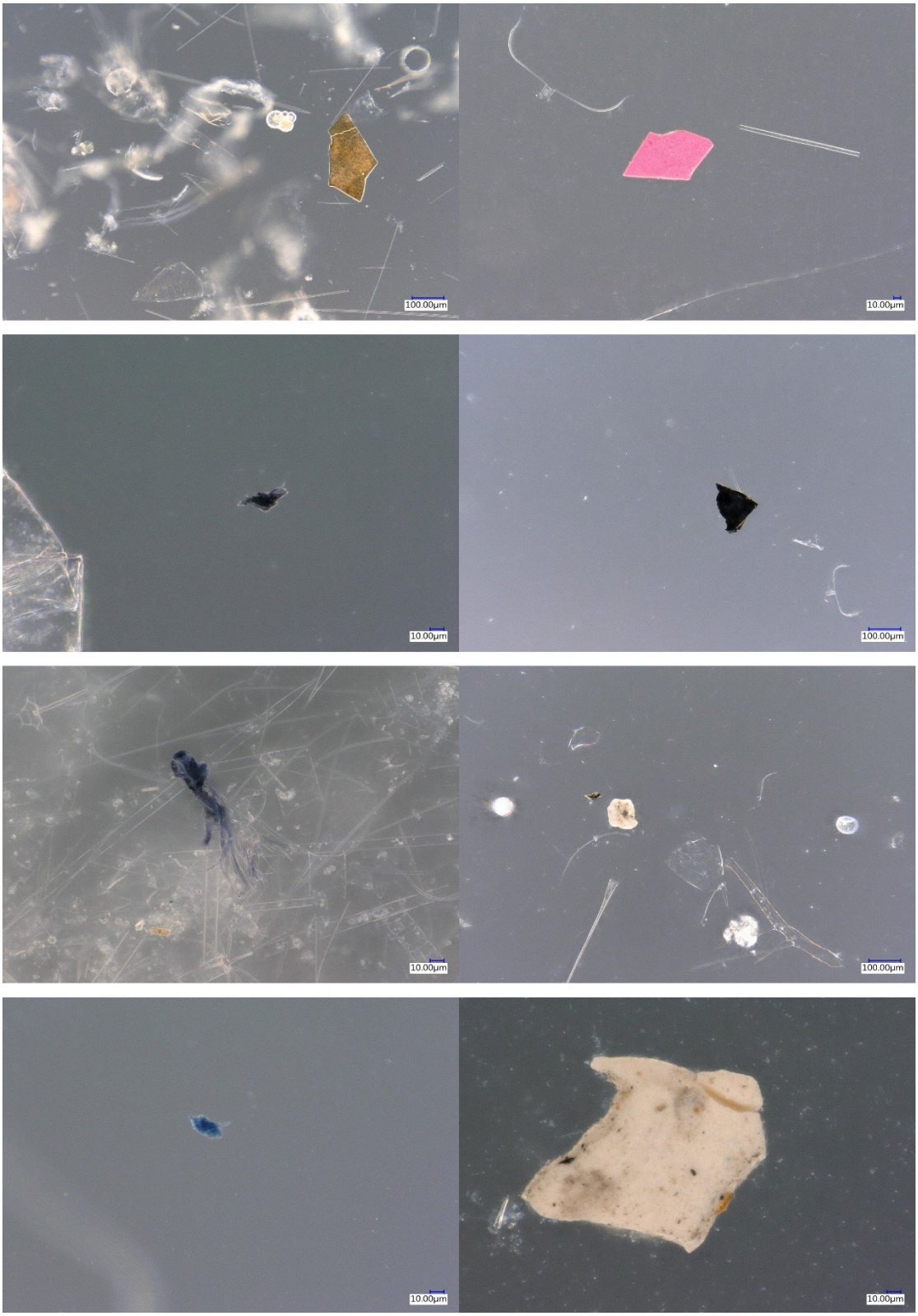


Figure 3.4.2. Although it is risky to determine whether a particle is plastic with only microscope photographs. The photographic representation of some particles that appear to be plastic and smaller than the mesh size may support the hypothesis to explain how the particles smaller than 64 µm detected in µ-FTIR analyses were captured.

CHAPTER IV: Microplastic detection in zooplankter gut using the digital microscope

Preface

The increasing amount of plastic pollution and its potential harm to living things were stated in Chapter I. Then, it was shown that plastic pollution is not only a problem of surface waters, but also covers the water column in Chapters II and III. In the present chapter, it was discussed whether the plastic, which has been proven to exist in the environment in chapters II and III, is ingested by zooplanktonic organisms or not. The surface of the whole body of each candidate zooplankter was included in the μ-FTIR analysis procedure after clearing all foreign particles with a high-resolution microscope, unlike similar studies. In fact, at the beginning of the study, the search for particles that look like plastic began as a challenge, focusing directly on the particles in the gut of zooplankter. However, no plastic-contaminated zooplankter was found at the results of the trials.

Introduction

The relationship between plastic pollution and living things has been known for a long time. Since 1969, when aquatic organisms were first reported to ingest plastics (Kenyon and Kridler, 1969), from fish to whales, turtles to octopuses, hundreds of species have been reported to ingest plastic (Lusher et al., 2017b). During this time, new plastic types were produced and included in pollution. Moreover, different components of each new plastic types, i.e., additional substances and their potential effects, have developed as another research subject. Thus, it is shown in some studies that the additives contained in plastics and harmful substances adhering to them from the surrounding environment may have a harmful effect on living things (Campanale et al., 2020). Although all animals have a role in the functioning of the ecosystem, some are essential for the system to stand together. For example, zooplankton are the first stop on the journey of the energy produced by phytoplankton, which converts solar energy into food by photosynthesis, to the top of the food pyramid (Sverdrup et al., 1942). In an *ex-situ* study, it has been proven that zooplankton transfer plastics, which are stowaways, along with energy transport to upper trophic levels (Setälä et al., 2014). Therefore, plastic ingestion of zooplankton endangers not only their own level but also the entire aquatic

system. Thus, the relationship and effect of plastic with zooplankton should be excessively examined.

It was shown in Chapters II and III that plastic pollution in the aquatic system does not only cover the surface of sediment but also in the water column. Thus, the presence of plastic in varying amounts and sizes in the habitats of zooplanktonic organisms in the water column from which the samples were collected was confirmed. Since there is both zooplankton and plastic in the environment, it was checked whether there was any contaminated zooplankton. The study was not limited to species or taxonomic groups in this analysis process. The possibility of the suspended plastic particles sticking to the zooplankton in nature and the sampling was considered. The study focused directly on the gut of individual zooplankter and investigated whether there were any suspected plastic particles.

Materials and methods

The study area and sample collection sections of the material method section of Chapter II are valid in the present chapter. For this reason, repetition is avoided in the text by not rewriting it here.

Sample preparation

The samples were kept in cool and opaque boxes, during the cruise. Then, 10% volumes were separated from the each samples delivered to the laboratory for this study with a Motoda splitter (Motoda, 1959). Then, each sub-sample was placed in vials suitable for their volume and kept for microscopy analysis in the dark room.

Microscopic analysis

Microscopic analyzes were performed with the Keyence VHX-6000, a high-resolution digital microscope. Before the analysis, the samples were taken into petri plate and mesoplankton and microplankton were separated for convenience in the next stages (This stage was shown in Figure 4.2.1.). Then, the mesoplankton were taken one by one and the microplankton were taken to the coverslip drop by drop and the gut content was checked. The gut of all individual zooplankter was examined separately. Zooplankter images were blended with 250x (combin-

ing with Keyence VHX-6100 camera unit and Keyence VH-Z250R magnification lens) magnification with stitching and live depth composition. Thus, a comprehensive image of the body was obtained as 3D. If any particles were found on the body, it was washed until it was completely cleared.

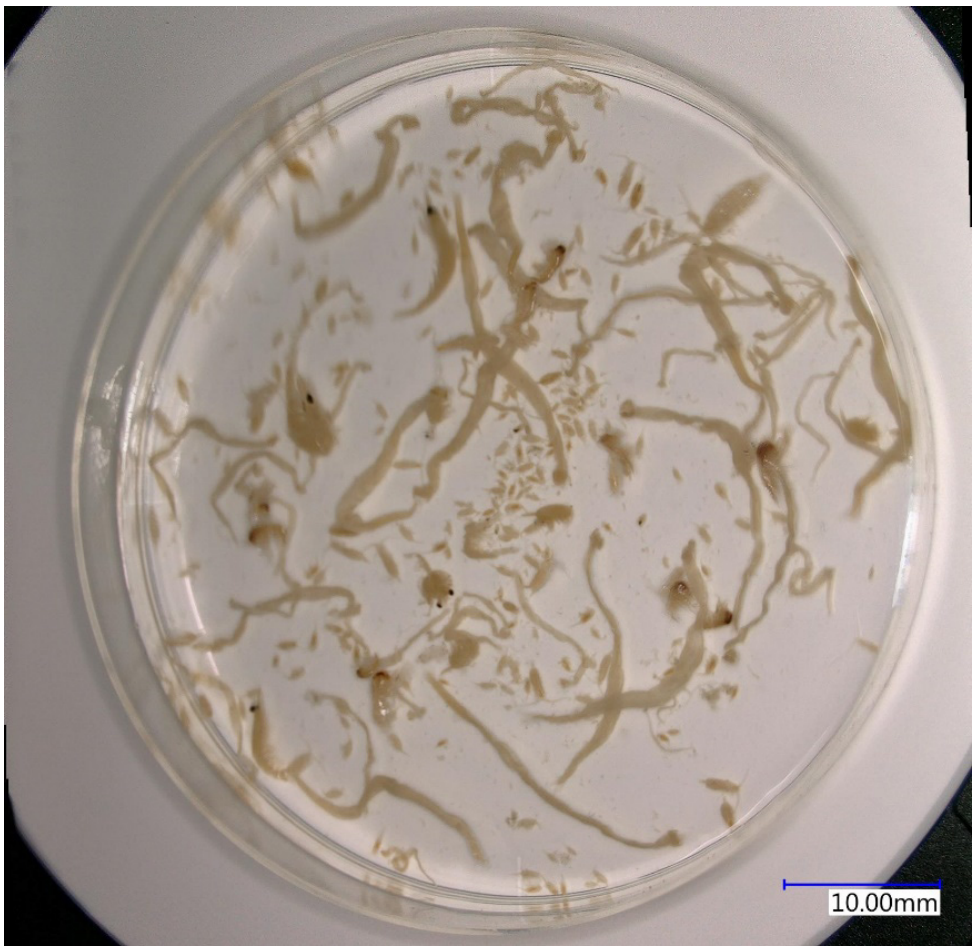


Figure 4.2.1. Separation of mesoplankton and microplankton to facilitate microscopic analysis.

After visual control, the zooplankter, a suspected plastic particle in the gut content, was transferred to the plastic detection analysis. Plastic identification analyzes were performed the same as in Chapter II.

Results and discussion

Thousands of zooplankton guts were analyzed with microscope, as mentioned. In addition, dozens of them were analyzed with FTIR. Nonetheless, no plastic-contaminated zooplankter was found.

The all zooplanktonic organisms in the sub-sample bottles were examined independently of species or groups. The skin and appendages of each zooplankter were examined with a high-resolution digital microscope and all foreign material on their surfaces was washed. The reason for such rigorous analysis was that carcasses, aggregates and detritus were blended with the targeted organisms in the sampling, as shown in Figure 4.3.1.

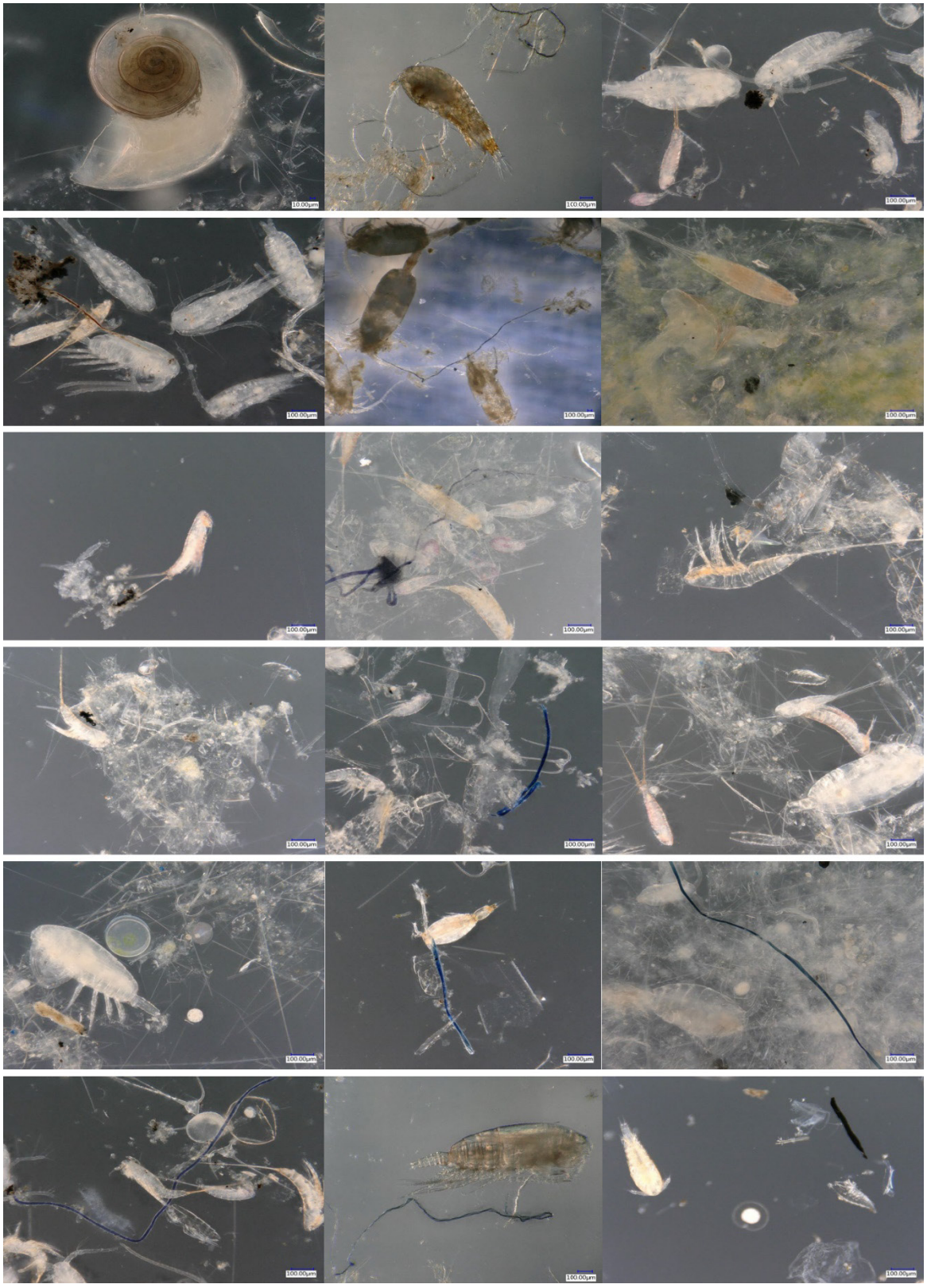


Figure 4.3.1. Blend of zooplankton with other water column members. The condition of zooplankton with other members before any treatment is applied to the specimens.

The aforementioned particles contaminated on the skin may play a key role in zooplankton-microplastic studies. While detecting with brightfield microscopes is quite difficult, it can only be detected with high magnification for darkfield microscopes. The skins of zooplankton before cleaning were shown in Figures 4.3.2. and 4.3.3.

Figure 4.3.2. An example of particulate contamination on the skin. Unknown particles on a copepod.

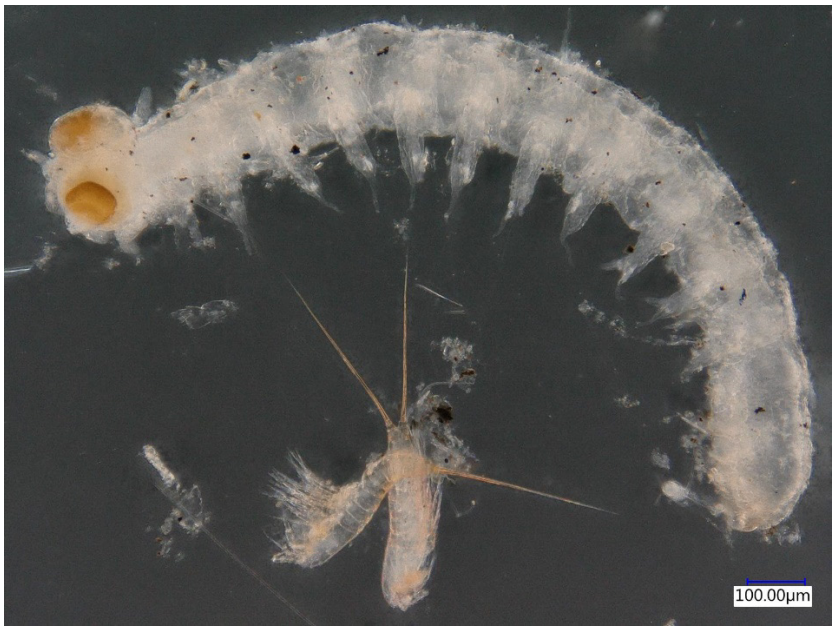


Figure 4.3.3. An example of particulate contamination on the skin. Unknown particles on a polychaeta.

The importance of high magnification was demonstrated in Figures 4.3.4. and 4.3.5. Low magnification was used in similar studies (Botterell et al., 2022). However, even these two examples show that zooplankton's skin surfaces can host contaminated particles.

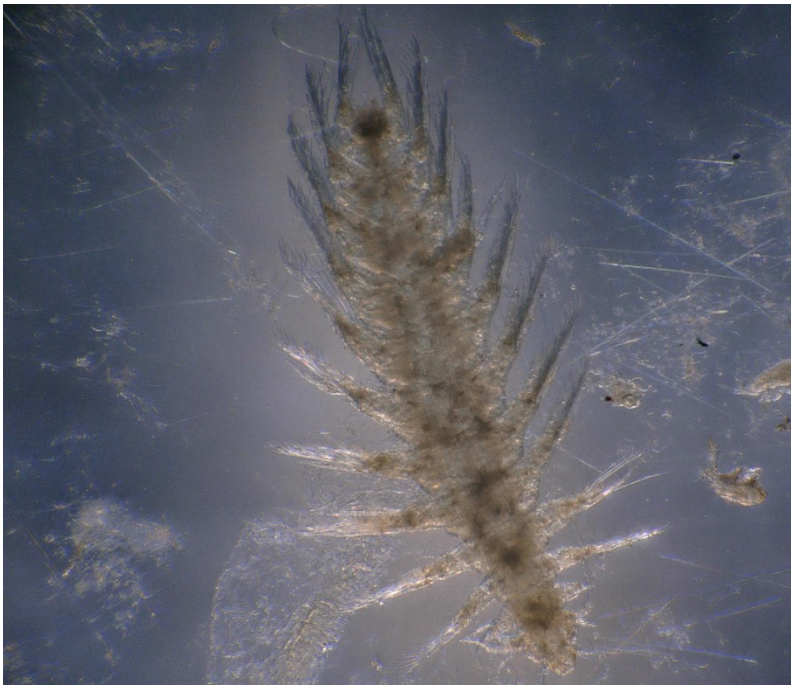


Figure 4.3.4. The appearance of a polychaeta at relatively low magnification. The particles on it are difficult to see.

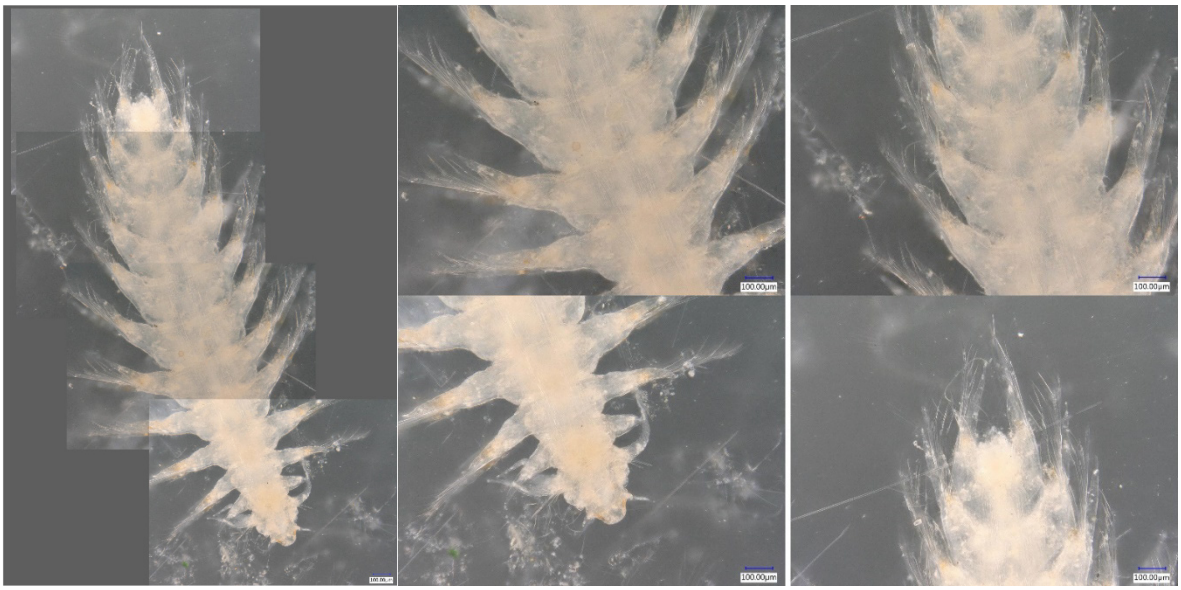


Figure 4.3.5. High magnification view of a polychaeta. Unknown particles of a size that FTIR can detect are visible.

Suspicious particles were even found attached to the skin of some zooplankton, but even these were not included in the FTIR analysis. As an example, Figures 4.3.6. and 4.3.7. show a fiber-like particle embedded in the skin of a copepod.

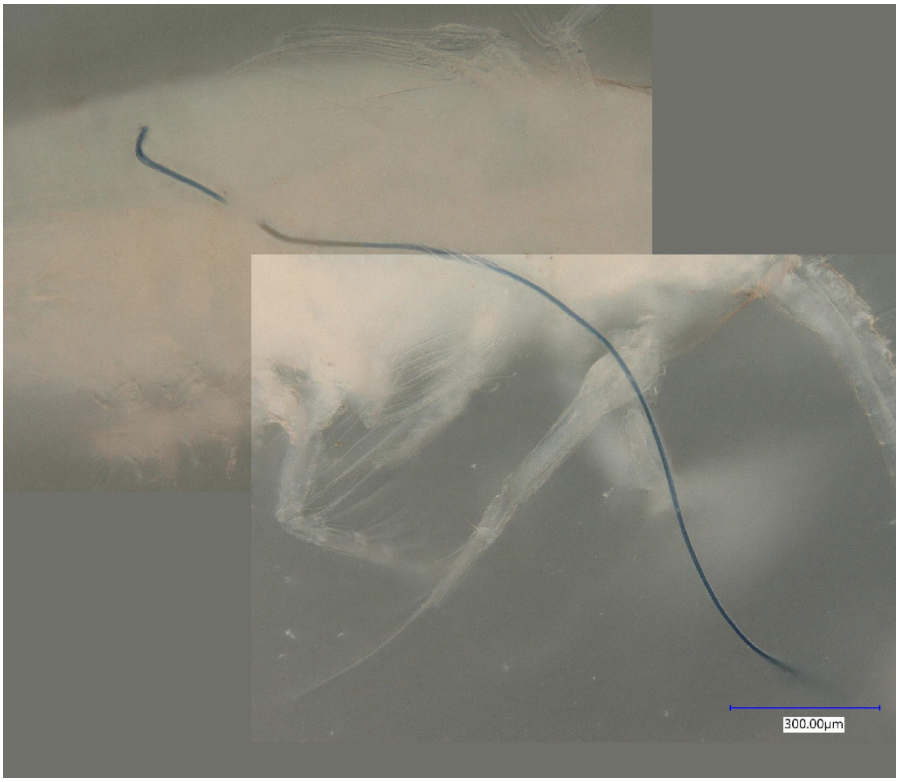


Figure 4.3.6. A fiber particle attached to the skin of a copepod.

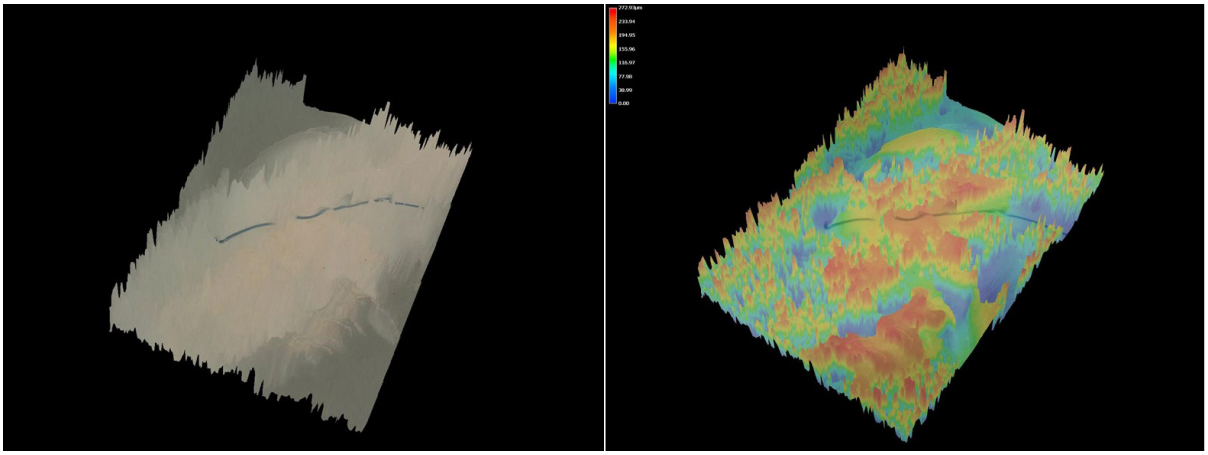


Figure 4.3.7. 3D visual representation of a fiber particle adhered to the skin of a copepod, with the fiber inside the shell.

Only completely cleared zooplankton samples were included in the FTIR analyzes as shown in Figures 4.3.8, 4.3.9, 4.3.10, 4.3.11. and 4.3.12. In addition, the cleaning of zooplankton was handled as sensitively as possible to preserve the gut contents.

Figure 4.3.8. The microscopic presentation of a zooplankter was included in the FTIR analysis. In addition, the body surface of the copepod in the image was completely cleaned.

Figure 4.3.9. The microscopic presentation of a zooplankter was included in the FTIR analysis. In addition, the body surface of the copepod in the image was completely cleaned.

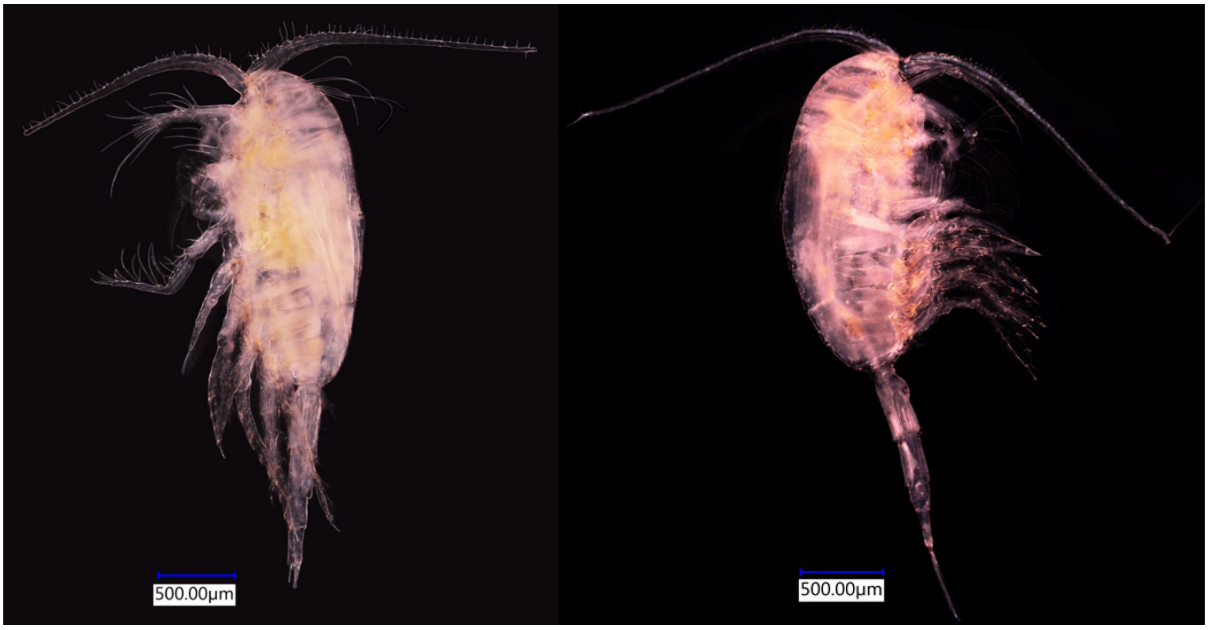


Figure 4.3.10. The microscopic presentations of a zooplankters were included in the FTIR analysis. In addition, the body surface of the copepod in the image was completely cleaned.

Figure 4.3.11. The microscopic presentation of a zooplankter was included in the FTIR analysis. In addition, the body surface of the copepod in the image was completely cleaned.

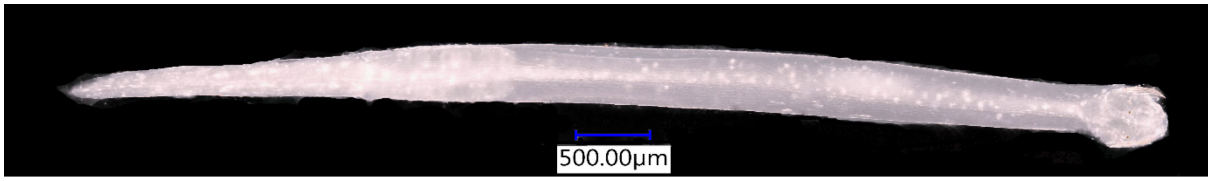


Figure 4.3.12. The microscopic presentations of a zooplankters were included in the FTIR analysis. In addition, the body surface of the chaetognath in the image was completely cleaned.

Although plastic particles in sizes that zooplankton could ingest were detected in the water column from which the samples were collected, no contaminated zooplankter was found. This may be because, unlike in other studies, the samples were treated meticulously. In other studies, the body surfaces of the samples were cleaned using low magnification, usually with brightfield microscopes. However, in the present study, all foreign material up to 5 µm in size was removed by using the darkfield technique with high magnification.

On the other hand, in some other studies, the samples were frozen and fixed. However, in the present study, it was fixed using 3% formalin. The effect of these different fixe methods on the guts of the specimens is unknown. Therefore, the effect of this difference on the results is not clear.

In addition, a significant part of the zooplankton in the sampling had lost their body integrity or had already become carcasses. So, for this reason, a part of zooplankton was directly eliminated. In addition, gelatinous organisms belonging to the zooplankton lost their forms that the effect of mesh sampling. As a result, no plastic-contaminated zooplankton was found in the gut. Finally, some photographs taken during the microscope analysis were added to the continuation of the text. These photographs also reflect the zooplankton that have lost their bodily integrity, are already in the carcass state, or are completely intact. The aforementioned photographs are presented as Figure 4.3.12. on the following six pages.

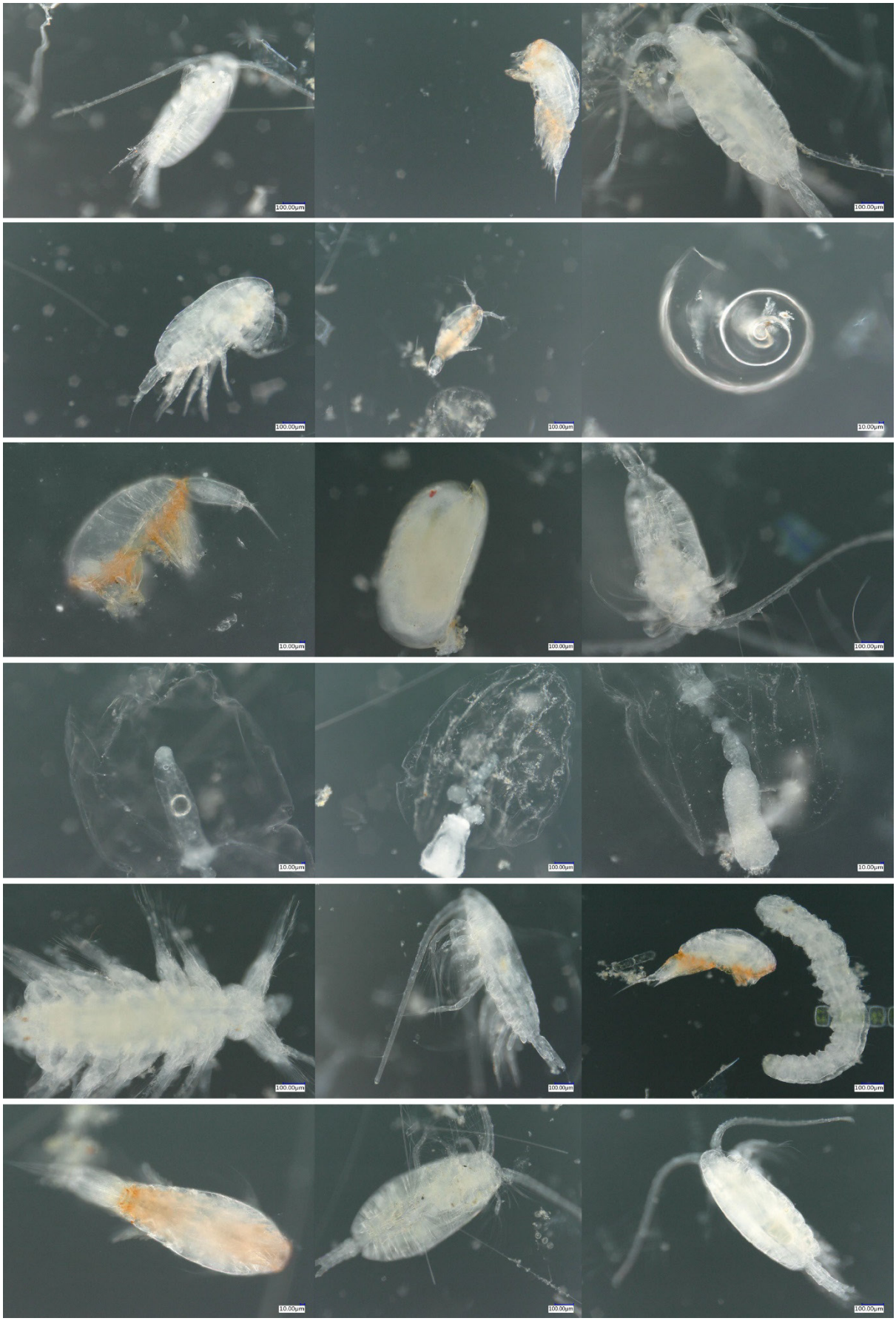
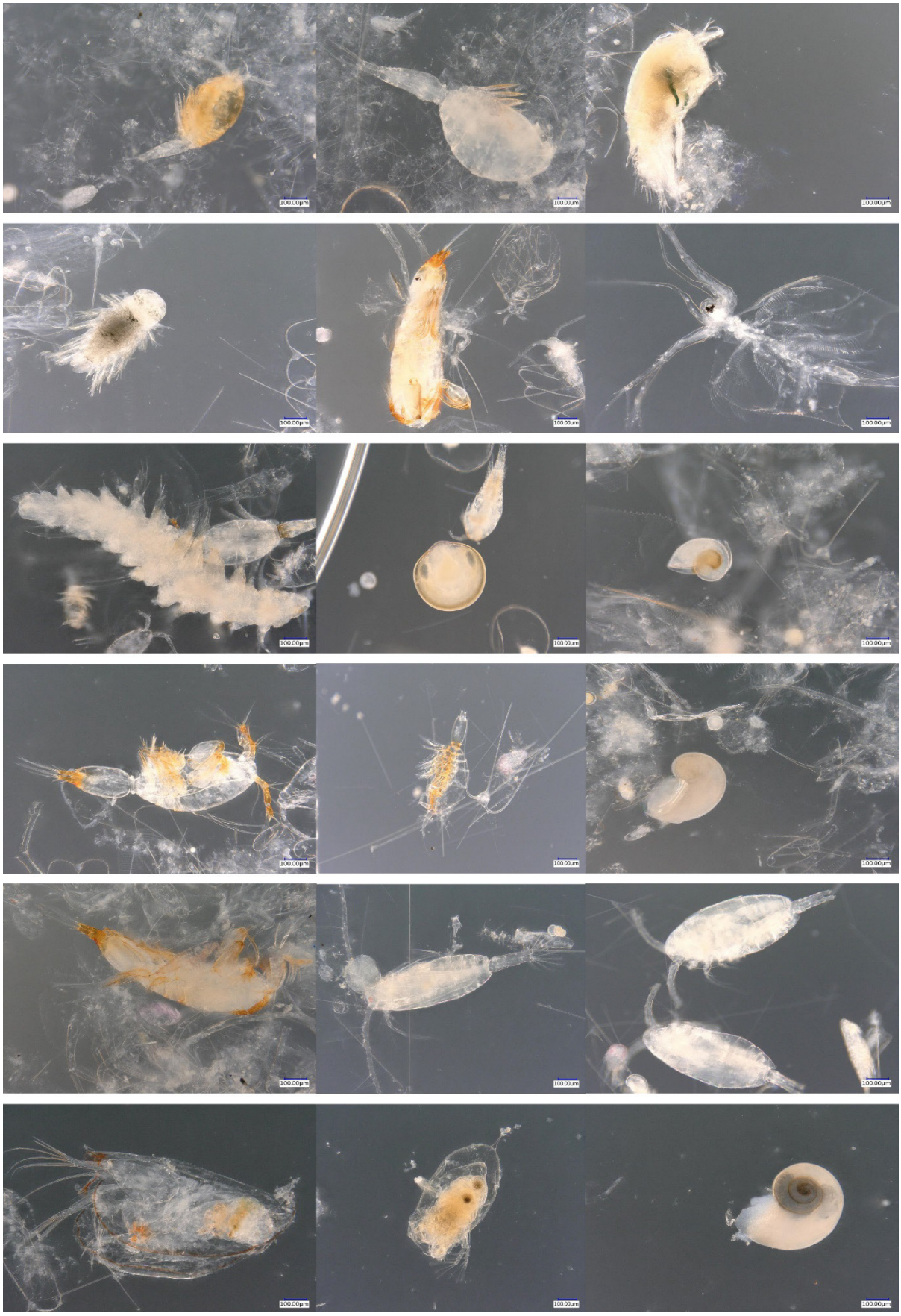


Figure 4.3.12. continued



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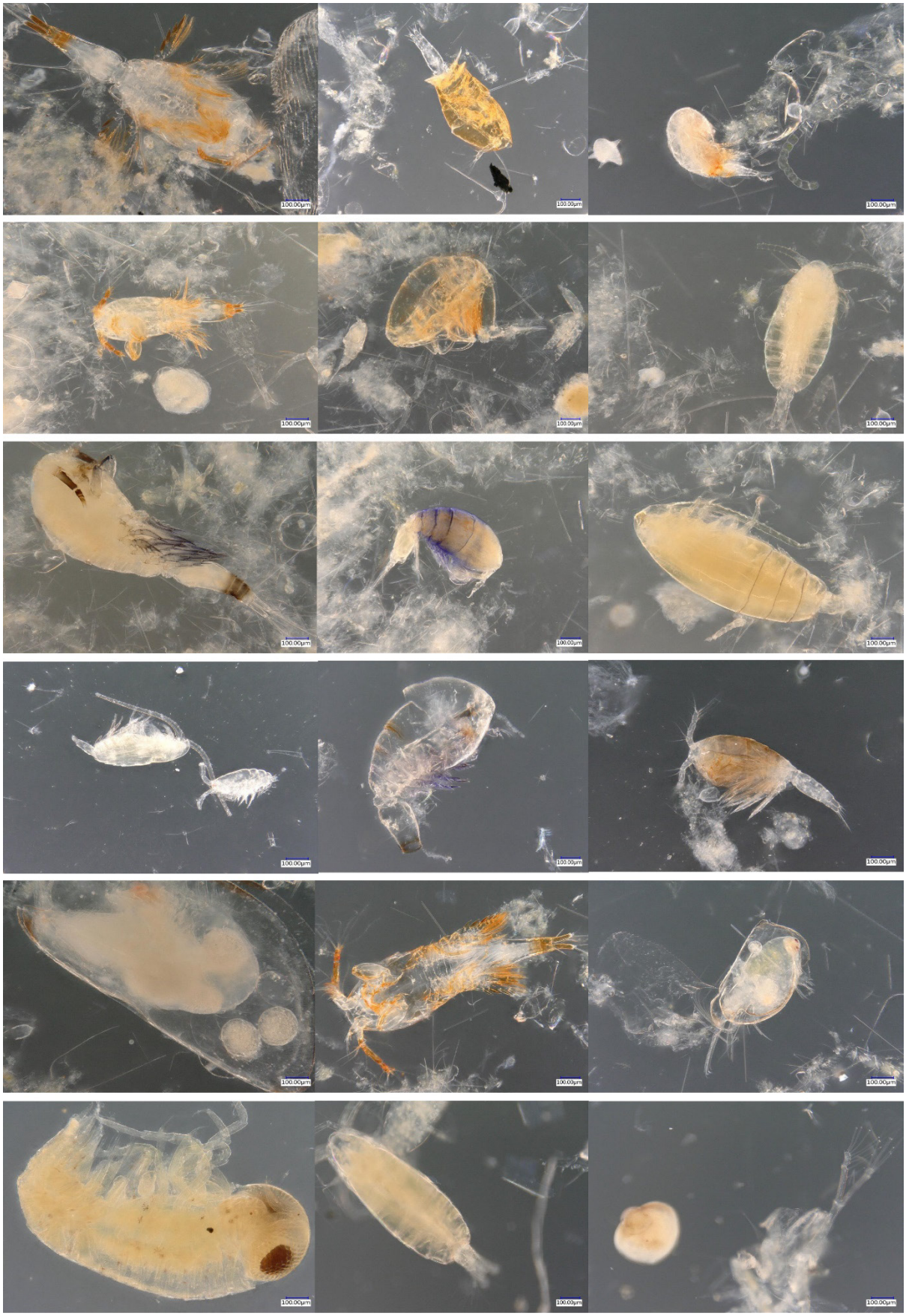


Figure 4.3.12. continued

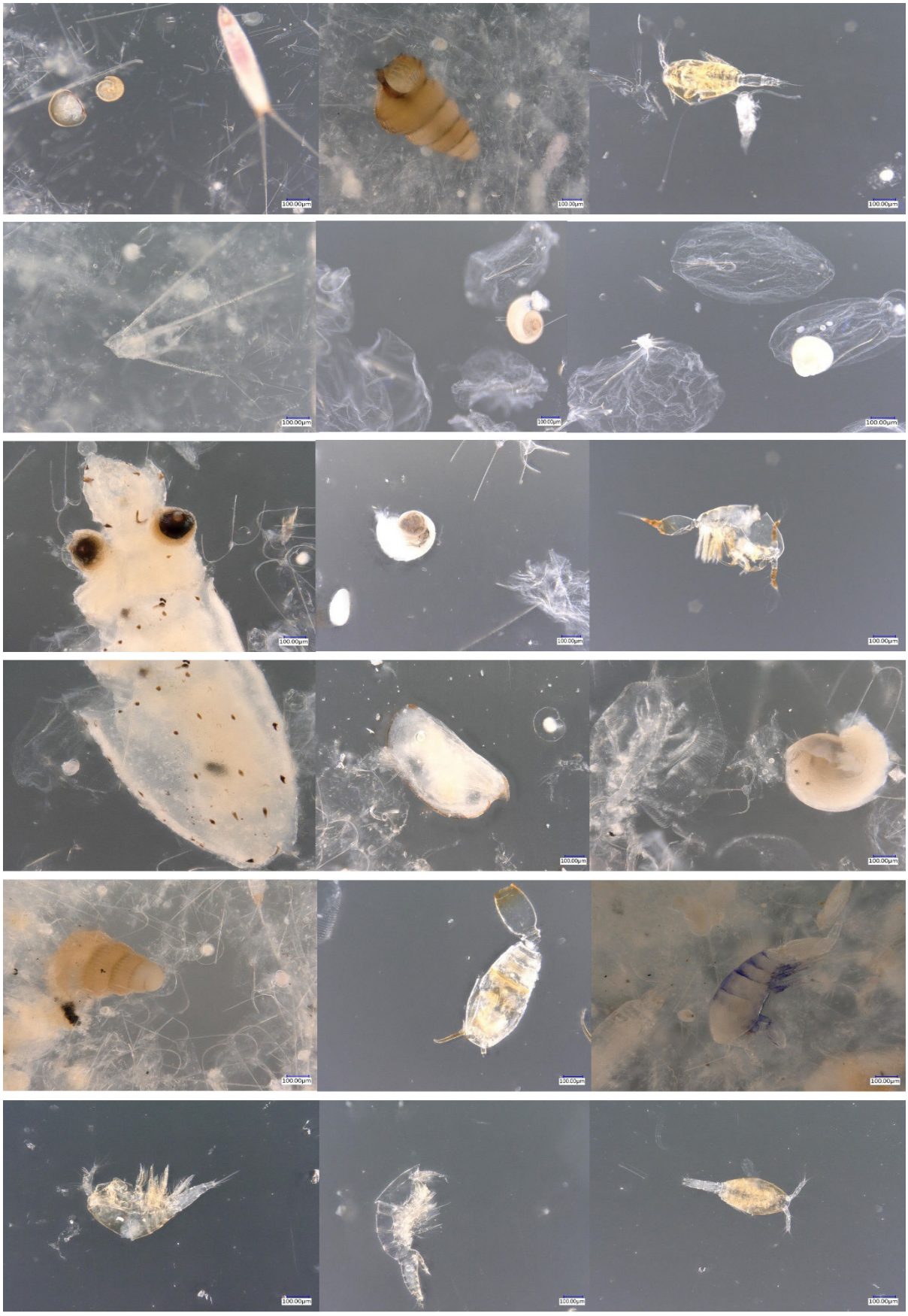
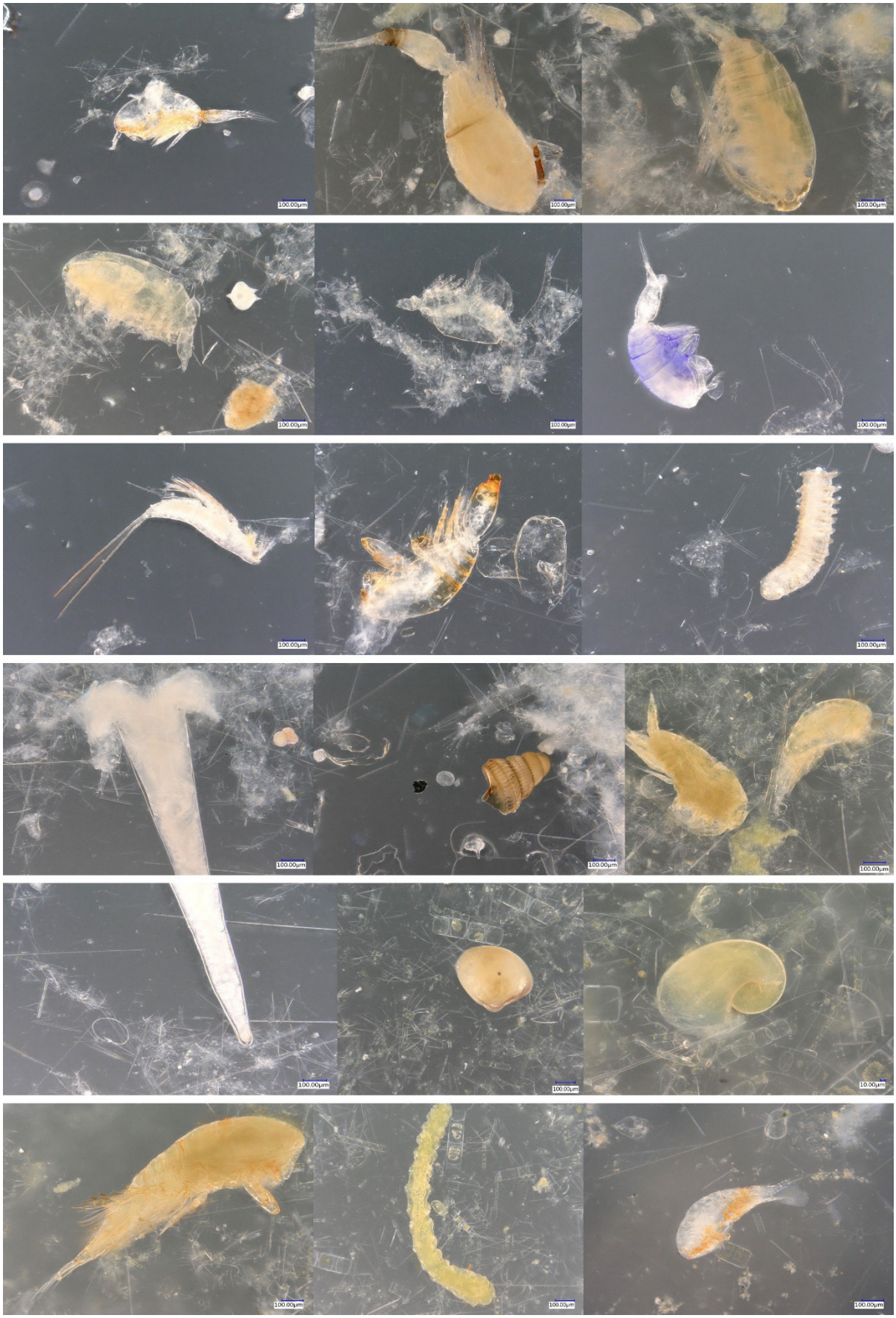


Figure 4.3.12. continued



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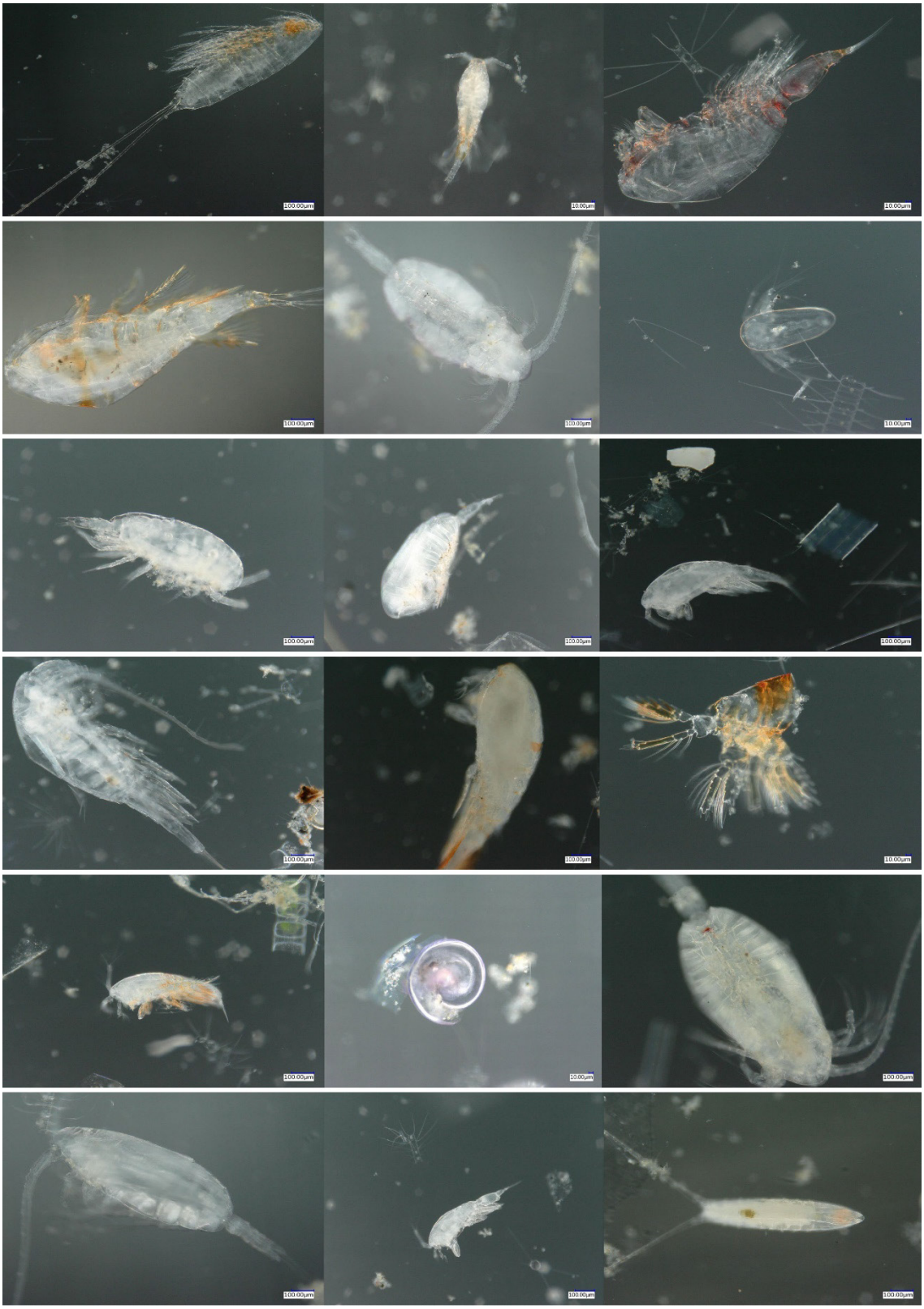

Figure 4.3.12. Photographs obtained as a result of microscope analysis of plankton sampling collected with the MOCNESS at three stations in the Sea of Japan. There are various zooplankton species in the photographs. While some of these zooplankters have preserved their body integrity, some have lost it.

CHAPTER V: Microplastic encapsulation capacity of zooplankton

Preface

Although no plastic-contaminated zooplankter was found in Chapter IV, it is known that zooplankton ingests plastic. Therefore, especially in *ex-situ* studies, it has been a research subject to investigate whether zooplankton ingests plastic and whether this affects the animal. Indisputably, the negative impact of a zooplankter due to plastic pollution is a critical and important issue. However, it is also important to learn how many plastics the zooplankton, which is one of the key points in the food chain extending to humans, can transfer to the next trophic level. Therefore, unlike in other chapters, an *ex-situ* study was carried out in the present chapter. The study focused on figuring out how many plastics a zooplankter can keep in its gut rather than how many plastics it can ingest. This result gives an idea of the amount of plastic a zooplankter can transfer from itself to the upper trophic levels. In zooplankton feeding studies, previously, how much food the animal consumed was found in a decreasing amount in the environment. However, in this study, the amount of plastic ingested by zooplankton from the environment was calculated from gut volumes. After determining the gut volumes with a digital microscope, the void volume between the plastic spheres was found with the Kepler conjecture. Therefore, only the volume occupied by the spheres was calculated. The application steps of this utterly novel method are explained in detail in the following text.

Introduction

Plastic production and consumption are increasing day by day, and its waste has become a worldwide issue in the aquatic environment (Jambeck et al., 2015; Lebreton et al., 2018). As known, plastic materials that are not solved *per se* have been accumulating and spreading *in natura* since the first day they were produced. Atmosphere, hydrosphere, lithosphere, and biosphere are already contaminated with plastic due to this spread, which is managed by natural and anthropological reasons (Brodhagen et al., 2015; Jambeck et al., 2015; Dris et al., 2018; Bergmann et al., 2019). While some types of plastic are recyclable, most types of plastic are either difficult or expensive to recycle. Therefore, the amount of plastic by 2050 may reach 25,000 Mt (Geyer et al., 2017).

Recently, there has been an increase in interest in the contamination of plastics smaller than 5 mm, called "microplastics (μPs)," in the aquatic system (Avio et al., 2017). These μPs are produced as primary (cosmetics, virgin, *etc.*) and secondary (broken from macroplastics) (Eerkes-Medrano et al., 2015). To scientifically understand the situation of μPs pollution in the aquatic environment, researchers now try to determine the amount of plastics in the oceans and even understand its influence on marine organisms (Cole et al., 2013). Indeed, plastic contamination at various stages of marine trophic levels has already been reported in various animals such as amphipods, annelids, aves, barnacles, bivalves, cephalopods, cetaceans, cnidaria, decapods, echinoderms, fishes, isopods, protists, reptiles, and various zooplankton species thanks to the result of these studies. (Lusher et al., 2017b).

It has been observed that a large part of the plastics in the marine environment originates from rivers (Wagner et al., 2014; Eerkes-Medrano et al., 2015; Schmidt et al., 2017). Dris et al. (2015) found that μPs exist in the water column for rivers 0.1–1 items m^{-2}, for lakes 10^{-4}–10^{-1} items m^{-2}, also sediment for lakes 10–10^{4} items m^{-2}, sediment for rivers 1–10^{3} items m^{-2}. Hence, the freshwater ecosystem is assumed to be affected by plastic contamination in various trophic levels including zooplankton. Therefore, to estimate how zooplankton are affected by μPs, quantitative information on the ability of zooplankton to encapsulate μPs particles is needed.

In the present experimental study to quantify the ability of μPs encapsulation by a *Daphnia manga* typical zooplankton in the freshwater ecosystem. In the present study, as a model plastic particle, I used microparticles of polystyrene (PS) one of the "big five" plastic-type (Plastic Europe, 2010), with the ecotoxicologically popular species *Daphnia magna* (Jemec et al., 2016; Frydkjaer et al., 2017; Imhof et al., 2017; Eltemsah and Bøhn, 2019; De Felice et al., 2019; Nakano et al., 2022), to understand the plastic encapsulation of zooplanktonic organisms, an essential link in the limnologic food web.

The present study tried to solve the following questions: (i) Does the presence of food in the environment affect the amount of plastic encapsulation of *D. magna*? (ii) Can *D. magna* distinguish the difference between plastic material and food? (iii) Can the plastic encapsulation capacity of *D. magna* be determined? (iv) Can the surface area of the encapsulated plastic material be calculated?

Materials and methods

Preparations and experiment

Daphnia magna culture

In the present study *Daphnia magna* used that is originally from National Institute of Environmental Studies, Japan (NIES). This animal was cultured in 500 mL glass beakers at 22 ± 1 °C in an incubator with artificial illumination (23.2 ± 0.4 µmol m^{-2} s^{-1}) as 16:8 h light: dark for many generations (Sebens, 1982). The culture was fed with unenriched micro-green algae *Chlorella vulgaris* (maximum concentration: 8×10^4 cells mL^{-1}).

Gut clearance of *Daphnia magna*

It was predicted that, if *D. magna* were starving for a too long time and fed afterwards, feeding behavior would be abnormal. Therefore, *D. magna* had taken in a food-free environment, and the gut situation was controlled every 1 h under the microscope until the main experiment was started. Before the experiment, I made sure that the gut of the *D. magna* fasted for 4 h was entirely cleared. I thus assume the optimum time to be 4 h. For this reason, the duration of the experiment was designed as 4 h when acute and significant changes occurred in the *D. magna* digestive system (Rosenkranz et al., 2009).

Microplastic solution

For the ingestion experiment, I used polystyrene microplastics (PsµPs) which are the most abundant plastic-type in the freshwater and marine environment (Li et al., 2016). The PsµPs (Polysciences Polybead *Ø* = 10 µm 1.05 g cm^{-3}, concentration; ~4.9×10^7 particles mL^{-1}) concentration was adjusted to for each microplate chamber within ~4.9×10^4 particles mL^{-1} (27 µg mL^{-1}) for the environment to which the *D. magna* would be exposed to during the experiment. In the *Daphnia* studies, an experiment medium was used in different concentrations up to 1,000,000 particles mL^{-1} (Phuong et al., 2016). On the other hand, experiments have also been carried out in mediums that use much fewer µPs particles and are closer to natural conditions (290 particles mL^{-1} 1% of the algae particles (Imhof et al., 2017). In the present experiment, I found this ratio correct in line with my aim to find the encapsulation capacity of

Daphnia magna. Giving excessive μPs particles is suffocating the *Daphnia* with μPs. Giving niggardly fewer particles can prevent the *Daphnia* from reaching the μPs easily.

Illumination of experiment

The experimental environment was illuminated with a double arm transmission (Nippon P. I. Co., PLG-2-500V) from a metal-halide light source (Nippon P. I. Co., PCS-UMX250 cold lighting system). The illumination of the experimental environment was set to the same level as the culture condition (23 ± 0.4 μmol m^{-2} s^{-1}). The light source was not used perpendicularly to avoid a negative effect on the feeding behaviors of the daphnids. Instead, the light was made to distribute homogeneously in the experimental environment by using a light cube. To prevent *D. magna* from reflecting light from the ground, the experiment was carried out using a copy stand (Nikon Repro-Copy PF-4). Figure 4.2.1. shows how these instruments are combined and used in the experiment. In addition, these experiments were conducted in a dark room to avoid being affected by other light sources.

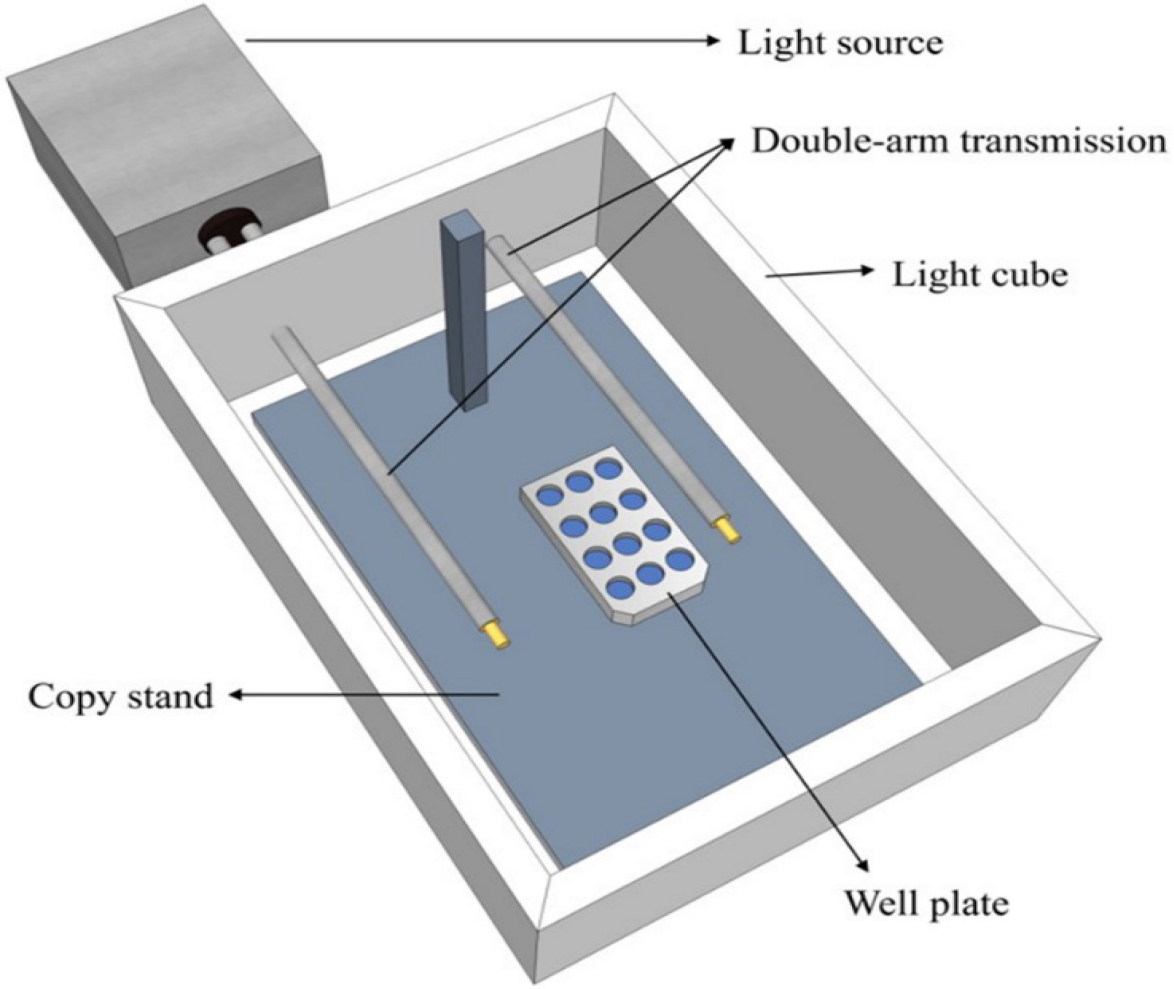

Figure 5.2.1. Schematic representation of the lighting system designed for the feeding experiment. The light from the light source was introduced into the light cube via the double arm and reflected on the inner surface of the cube. In this way, the light in the space limited by the cube was made homogenously diffused, so the experiment could be done without direct radiation on the animals.

Experimental environment

The experiment was carried out in six different conditions:

(i) “Control Condition” with only tap water without plastic or *Chlorella vulgaris*,

(ii) “10 µm µPs Condition” with tap water (24 h waited before use for dichlorination) and 10 µm µPs microspheres,

(iii) “Odor Condition” the *Chlorella vulgaris* culture was sieved with a GF/F 47 mm filter (pore size: 0.7 µm) (Whatman plc., Kent, U.K.). Removing particles larger than 0.7 µm from the environment and the remaining picoplankton, the use of the cultural fragrance was ensured,

(iv) “Odor & 10 µm µPs Condition” In condition odor, additional µPs added,

(v) “*Chlorella* Condition” kept same concentration with culture,

(vi) “*Chlorella* & 10 µm µPs” In condition *Chlorella*, additional µPs added (Table 5.2.1.).

For each condition, a 12-chamber well plate (well size 22 mm *Ø*, 17.5 mm H) was used; one *D. magna* was placed in each chamber. In this way, each condition was made in 12 repetitions.

Table 5.2.1. Design of experimental environment (● included, ○ excluded).

Conditions/Content	**µPs**	***Chl* Odor**	***Chl***
Control	○	○	○
***Chl* Odor**	○	●	○
Chl	○	○	●
10 µm µPs	●	○	○
***Chl* Odor & 10 µm µPs**	●	●	○
***Chl* & 10 µm µPs**	●	○	●

Experimental analyses

After this 4 h feeding experiment, the animals were gently washed for five seconds in a 2% formalin-water solution with the help of a Pasteur pipette. After washing, they were quickly examined for gut volume and plastic concentration in the gut under the a digital microscope (Keyence VHX-6000). After this process, it was fixed in the same formalin solution.

Microscopic analyses

Microscopic analyses were performed with the digital microscope. In the present study, thanks to the recent technological developments, the measurability has been made to the µm-scale volume calculations. *Daphnia* images were blended with 250x (combining with

Keyence VHX-6100 camera unit and Keyence VH-Z250R magnification lens) magnification with stitching and live depth composition.

Gut volume

The gut volume was measured using the cylinder volume formula

$$V = \pi r^2 h \tag{5.1},$$

where *r* is radius and *h* is height.

by taking the gut's length and arithmetic mean of width.

$$\bar{X} = \frac{1}{N}\sum_{i=1}^{N} x_i \tag{5.2},$$

where $\bar{X}$ is the arithmetic mean, N is the number of values and x_i is data set values. The gut system of *Daphnia magna* consists of the esophagus, midgut, and hindgut. Since the thicknesses of these three parts are different from each other, the average was taken by measuring from different points on the gut. In addition to the average gut thickness, the gut of *D. magna* is curved. The calculation of the length and thickness of the gut in this curved structure was shown in the Figure 5.2.2.

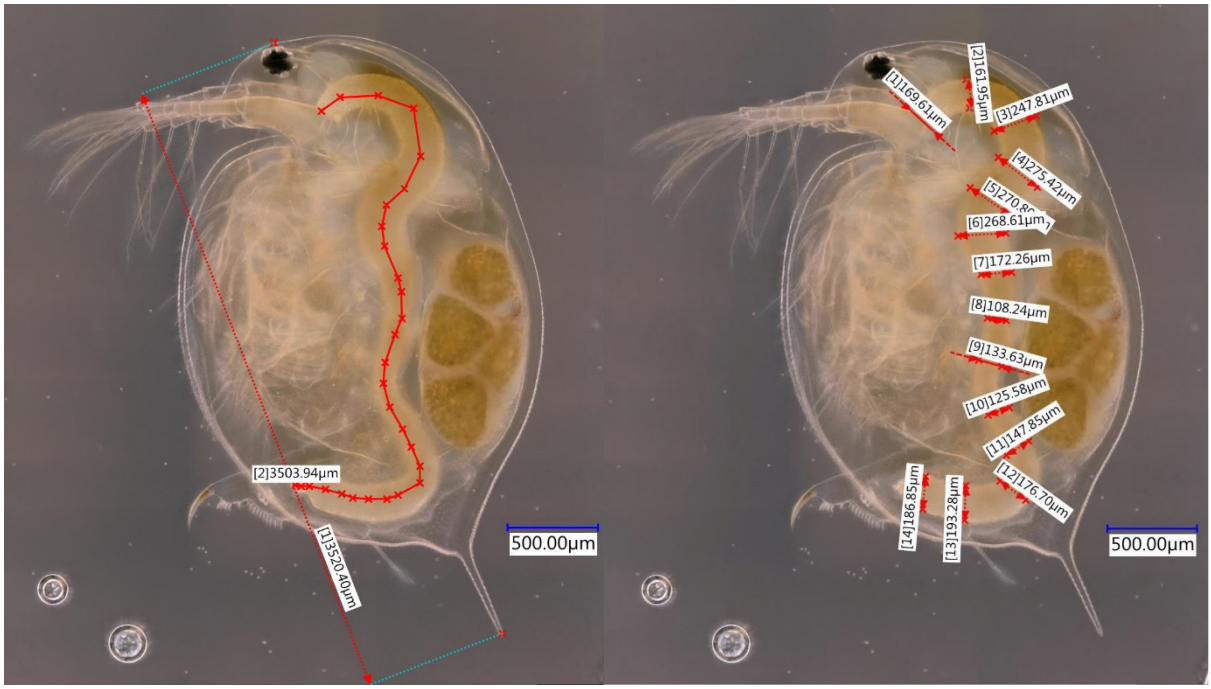

Figure 5.2.2. Demonstration of calculating the body length, gut length, and average gut thickness of *Daphnia magna* from a microscope photograph.

Number of encapsulated microplastic particles

Kepler conjecture (Hales, 2005) was chosen among various theories contention on for cannonball problem. The encapsulation volume was calculated by the Kepler conjecture formula since the volume of all µPs spheres is equal (FCC: Face-Centered Cubic, HCP: Hexagonal Close-Packed).

$$\eta_{\text{Kepler}} = \eta_{\text{FCC}} = \eta_{\text{HCP}} = \frac{\pi}{3\sqrt{2}} \cong 74.048\% \quad (5.3),$$

The surface area of encapsulated microplastic particles

The µPs spheres' surface area in the gut was calculated by determining the number of plastic particles encapsulated

$$A = 4\pi r^2 \quad (5.4),$$

where A is surface area and r is the radius.

Statistical analyses

Free statistics program R was used for graphs and analysis of One-Way ANOVA and Tukey post hoc test (R Core Team, 2022). Because of *Daphnia*'s nature, all individuals have different body sizes; therefore, a standard must be set first. Accordingly, a ratio can be estimated between the gut volume and overall height. From this ratio, calculated for the gut volume per 100 µm height is calculated. Thus, a general standardization was provided for each individual. Thereafter, the encapsulation data were prepared for statistical analysis.

Results

Gut volume and capacity

Images of each individual *Daphnia* were taken under the microscope. The *D. magna* guts of each of the µPs conditions were fully filled with µPs, which can be easily seen from the images (Figures 5.3.1. and 5.3.2.). The space between the spheres was found to be minimized by being stuffed in the gut. The motion of the gut stuffing the µPs was as shown in Figure 5.3.3, which is a series of time-lapse images taken every 2 s. From the images, the gut volume calculated from the images was calculated separately for each individual, and then the arithmetic means of six different experimental conditions in total (Table 5.3.1.).

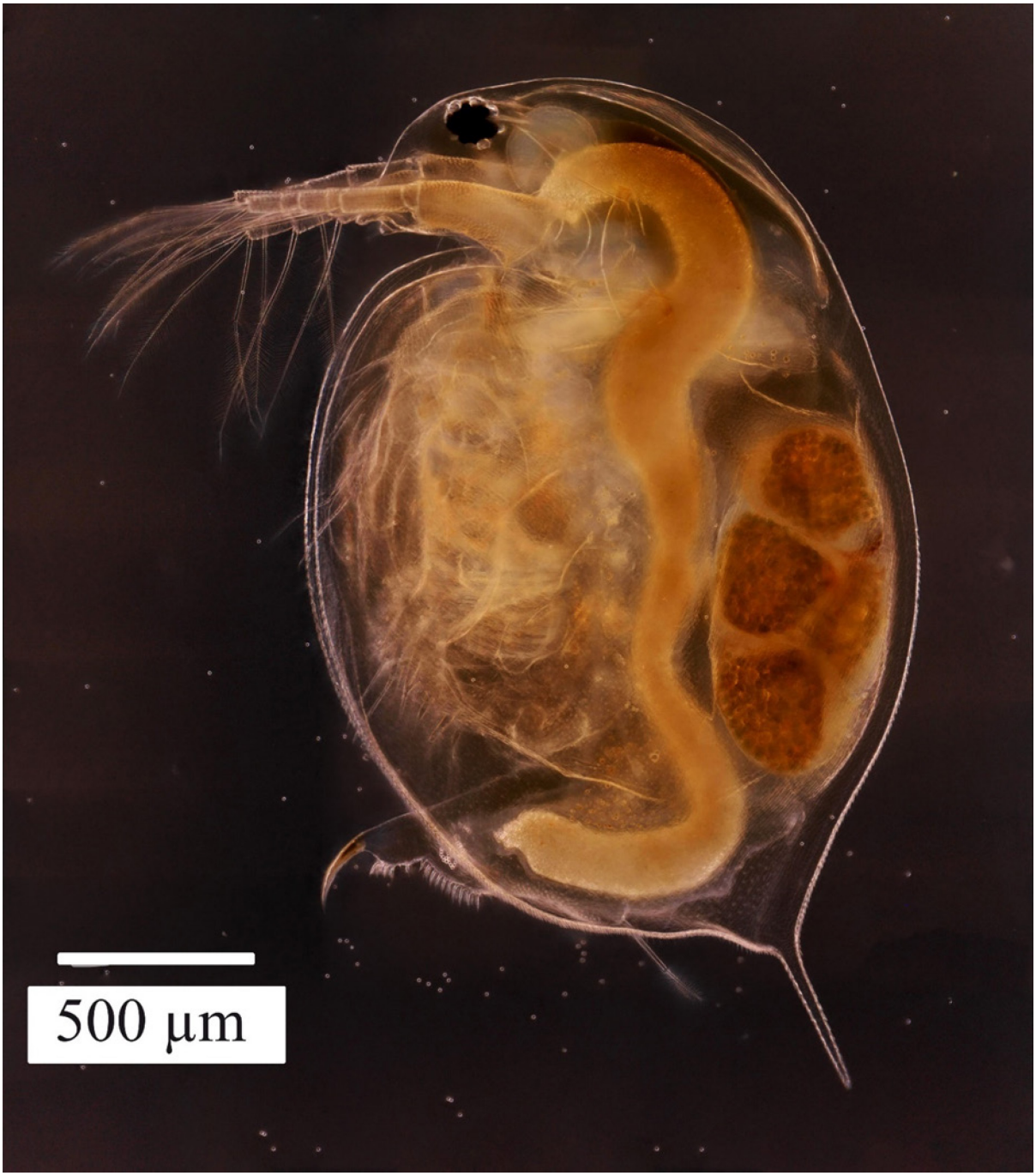

Figure 5.3.1. *Daphnia magna* with a microplastics-filled gut emerged after a 4 h feeding experiment.

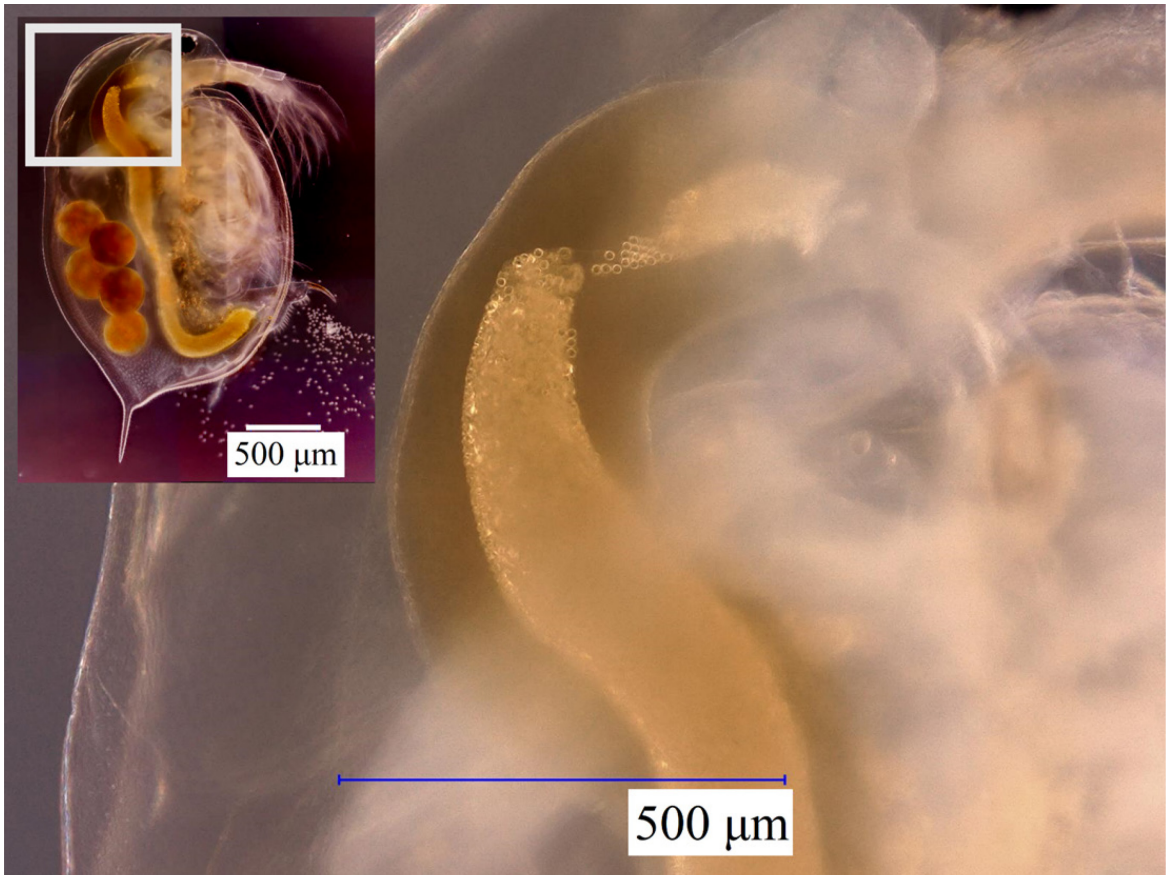

Figure 5.3.2. *Daphnia magna* gut filled with microplastics of 10 µm diameter.

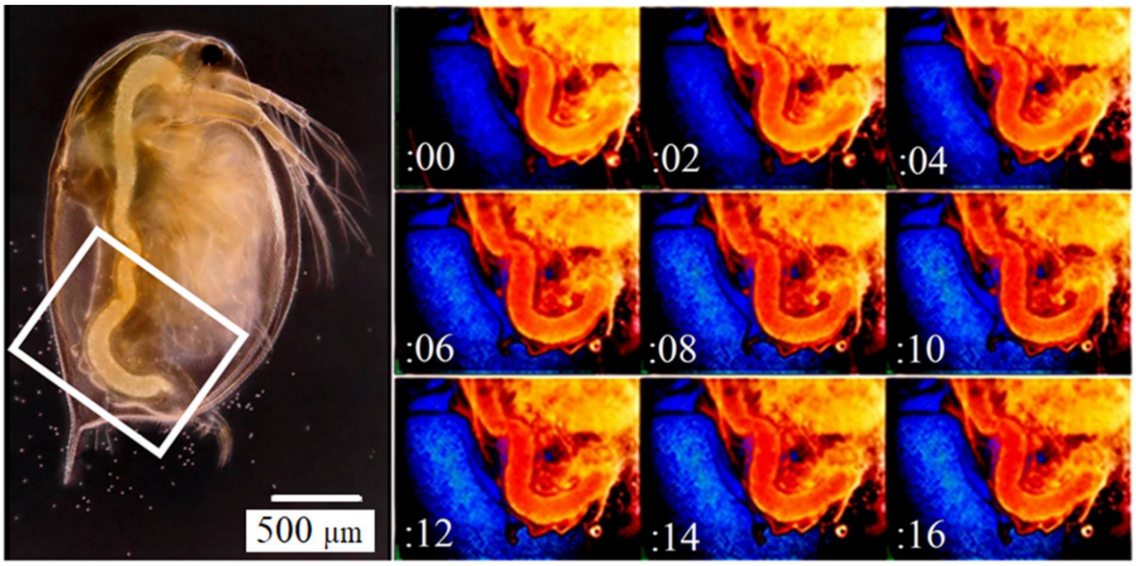

Figure 5.3.3. Every 2 s time series of the gut movement of *Daphnia magna* stuffing microplastics.

Table 5.3.1. Gut volume (μm^3) for each $\bar{X}$ 100 μm ($\bar{X}$: arithmetic mean) and ~4 mm body length.

	Control	***Chl* Odor**	***Chl***	**μPs**	**μPs *Chl* Odor**	**μPs *Chl***
~$\bar{X}$ 100 μm	1.8×10^6	2.6×10^6	3.9×10^6	4.0×10^6	2.7×10^6	3.1×10^6
~4 mm	7.2×10^7	10.9×10^7	15.9×10^7	16.0×10^7	10.9×10^7	12.5×10^7

Gut volume differences of conditions

There were highly significant differences in the gut volume between all groups (ANOVA test, $F = 6.127$, $p = 0.000101$). In the gut volume estimations, the condition with only μPs (d) dominates the peak point (μPs (d) between Control (a) $p = 0.0003741$). Another critical point is that μPs' effect on Odor conditions (b) and *Chlorella* conditions (e) were not statistically significant differences (*Chlorella* Odor (b) between μPs *Chlorella* Odor (e) $p = 0.999$, *Chlorella* (c) between μPs *Chlorella* (f) $p = 0.510$) (Figure 5.3.4.). I found no significant differences in the feeding volume of *D. magna* whether there were plastics in the feeding medium or not (*Chlorella* Odor (b) between μPs *Chlorella* Odor (e) $p = 0.999$, *Chlorella* (c) between μPs *Chlorella* (f) $p = 0.510$). Likewise, no statistical difference between plastics and phytoplankton for *D. magna* feeding was noticed.

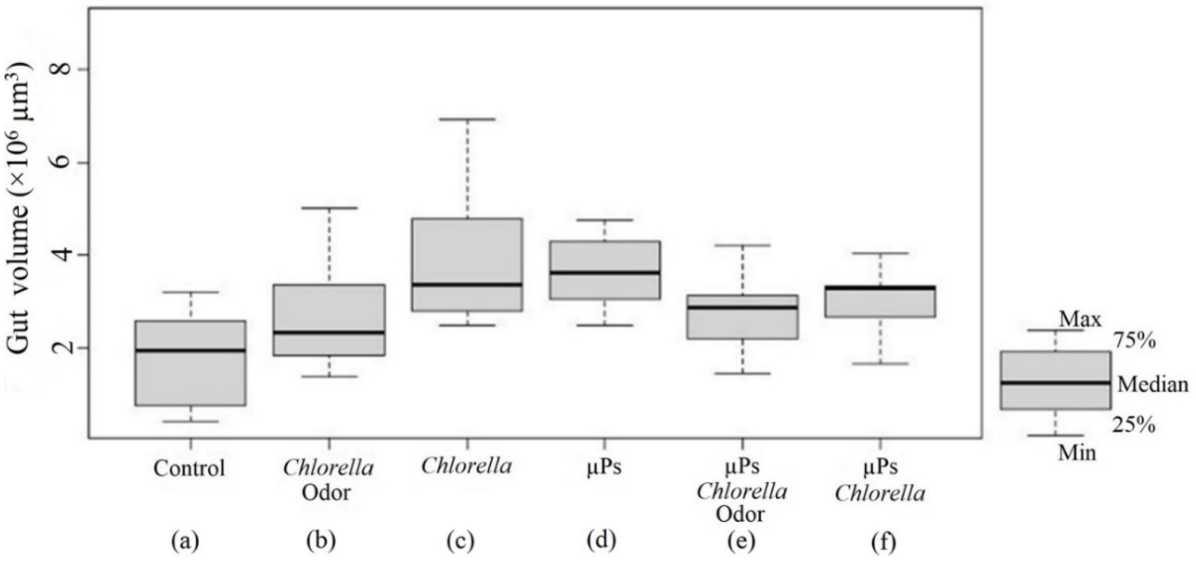

Figure 5.3.4. Gut volume differences by six medium conditions, arithmetic mean, high-low level, and median of gut volume difference for each 100 μm body length.

Number of encapsulated microplastic spheres

According to the Kepler conjecture, the space between the spheres occupies 25.952% of the volume of the object in which the spheres have located. Based on this assumption, it was thought that the remaining 74.048% volume belonged only to the spheres (Eq. 5.3). The volume of the 10 µm *Ø* sphere can occupy 5.23×10^2 µm^3 ($V_{10\ \mu m\ Ø\ sphere}$). was calculated using the Kepler conjecture formula, and the approximate number of spheres was calculated. The number of encapsulated plastics is shown in Table 5.3.2.

Table 5.3.2. Approximate numbers of encapsulated microplastic spheres (10 µm *Ø* sphere).

	µPs	**µPs *Chl* Odor**	**µPs *Chl***
~$\bar{X}$ 100 µm	0.56×10^4	0.38×10^4	0.44×10^4
~4 mm	26.50×10^4	15.55×10^4	17.81×10^4

The surface area of encapsulated microplastic spheres

The total surface area ($A_{10\ \mu m\ Ø\ sphere} \approx 3.14 \times 10^{-4}$ mm^2) of the number of spheres were calculated by use of the encapsulated µPs numbers The surface areas of the encapsulated µPs obtained from the µPs numbers are shown in Table 5.3.3.

Table 5.3.3. Approximate surface area (mm^2) of encapsulated microplastic spheres (10 µm *Ø* sphere).

	µPs	**µPs *Chl* Odor**	**µPs *Chl***
~$\bar{X}$ 100 µm	1.77	1.22	1.39
~ 4 mm	71.1	48.8	55.9

Discussion

The primary goal was to quantify how many µPs particles a single *Daphnia magna* can catch, rather than to understand the possible effects of plastics on this animal. Results shown here suggest that the surface area of µPs particles is important as well as their volume. It is because the surface area of a unit volume of µPs increases in inverse proportion to the size, and because, since they are not decomposed in nature, the number of plastics increases. Indeed, µPs of small sizes that are edible to zooplankton are suspended in the ocean in high concentrations not only in the surface layer but also in the deep layers. Therefore, I here stress the importance of the future study of µPs *in situ* and *in vitro*. Also, the findings in the present study that zooplankton could indiscriminately ingest 10 µm-sized µPs suggests the importance to further consider (1) if plastics themselves affect plankton growth, survival, and reproduction, and (2) how chemical substances adsorbed on the plastic surface could be bioaccumulated through the aquatic food chain.

Plastics may cause physical damages to the guts, congestion, and reducing the absorption of nutrients through the gut wall (Wright et al., 2013). In addition, plastics also may have chemical effects. Rochman (2013) has pointed these chemicals originate from the plastic itself (bisphenol A: BPA, polybrominated diphenyl ethers: PBDEs, phthalates, lead: Pb, polycyclic aromatic hydrocarbons: PAHs, styrenes, *etc.*) and are absorbed from the environment (Pb, PAHs, polychlorinated biphenyls: PCBs, nickel: Ni, PBDEs, *etc.*). Chemicals from the environment adhere to the plastic surface. When any organisms ingest plastics with such chemicals, the surface area of the plastics is of crucial importance. This is the exact reason why I investigated the encapsulation capacity in the model zooplankton *Daphnia magna*.

Since studies on *D. magna* have been made for a long time, there are various findings and definitions. According to some studies, *D. magna* is an entirely non-selective filter feeder (Weltens et al., 2000) or a selective animal that can partially identify food (Kirk, 1991). Likewise, the particle size *D. magna* can eat is known to be 1–50 µm (Ebert, 2005). However, it has been observed that this animal could capture particles as small as 24 nm using active filters (Geller and Müller, 1981). It has also been observed that it can ingest particles down to 20 nm (Besseling et al., 2014). Generally, µPs studies for *D. magna* have also been carried out in the scales identified by these references. Experimental results using various sizes of µPs such as 20 nm (Schür et al., 2019), 100 nm (Rist et al., 2017), 1 µm (De Felice et al., 2019; Schür et al., 2019), 2 µm (Rist et al., 2017), 1–5 µm rank (Ogonowski et al., 2016), 6

μm (Eltemsah and Bøhn, 2019), 40 μm (Imhof et al., 2017), have already been published. Focusing on the capacity to encapsulate under optimum conditions, I decided to study with 10 μm of particles between 1–50 μm, which is considered ideal for providing a clear view for the gut and using particles as small as possible (Figure 5.3.2.).

The capacity of mono-sized spheres to pack into a cylindrical form has been a matter of discussion for a long time. It is examined in two main elements as space occupation coefficient and void fraction. Beginning with Johannes Kepler (1611), it has attracted the attention of many researchers until today. For instance, Kepler suggested that spheres fill the cylinder volume at most 0.74048, and Hales (Hales, 2005)confirmed this in 1998. In 1955, this space occupation coefficient increased to 0.827 (Rankin, 1955) and reduced to 0.7797 only after three years (Rogers, 1958). Another perspective is that the spheres are randomly packed into the cylinder. In this regard, it is about 0.6366 ± 0.0005 for close packing (Scott and Kilgour, 1969), but also 0.64 ± 0.02 (Berryman, 1983). For granular matters, the space occupation coefficient has been found 0.608 ± 0.006 to random loose packing (Scott, 1960), and 0.555 ± 0.005 for zero gravity force (Onoda and Liniger, 1990). The coefficient rate in random packing; may increase with humidity (due to friction between spheres (Vandewalle et al., 2012), tapping (Berg et al., 1969), shaking (Berg et al., 1969; Scott and Kilgour, 1969), and vibration (Berg et al., 1969; Rocke, 1971; Tingate, 1973). Especially in Berg et al. (1969), the fact that 3D shaking can form an almost hexagonal close packing shape has been the determining factor for the present study. Because it has been assumed that the plastic sphere inside the gut takes a hexagonal close packing shape with 3D stuffing by the gut movements (Figures. 5.3.2. and 5.3.3.). Also, since both and the inside of the gut and the medium are liquid, the friction force between the spheres can be neglected (the ratio that should be the minimum may be 45%; Vandewalle et al. (2012). For these reasons, the ideal close packing rate of the present study was determined as the Kepler conjecture rate.

Using the Kepler conjecture, the number of plastics in the gut was calculated. For an encapsulation case if 4 mm length *Daphnia*, the μPs *Chlorella* condition, which is the closest condition to its natural environment than other media, was considered. The amount of encapsulated microplastic (*Ø* 10 μm) particles calculated for this condition was 17.81×10^4 (Table 5.3.2.).

The absorption and release of these chemicals by plastic particles directly depend on the volume and surface area. By determining the number of spheres that can be encapsulated, I can

calculate the surface area of the spheres that could react with the biota and enzymes in the gut. The number of possible encapsulated particles varies depending on the sphere diameter. This way, it was unveiled how the surface area changed according to the particle size (Table 5.4.1.). Although the plastic size in nature is not fixed or is not as dense as in the experimental environment, it is not unusual for organisms to be exposed to and encapsulate such plastic. For clarity, *Daphnia magna* (in case feeding with *Ø* 20 nm nPs) can encapsulate almost two soccer balls (*Ø* 220 mm, $A = 1.7 \times 10^5$ mm^2) in 4 h.

Table 5.4.1. Approximate surface area (mm^2) of encapsulated microplastic spheres for diet range of 4 mm *Daphnia magna*.

Particle diameter (µm)	**Surface area of µPs spheres (mm^2)**		
	µPs	µPs *Chl* Odor	µPs *Chl*
0.02	3700	2600	2900
0.1	71	45	56
1	7.2	4.9	5.6
5	1.4	1.0	1.1
10	0.71	0.49	0.56
20	0.36	0.24	0.28
30	0.24	0.16	0.19
40	0.18	0.12	0.14

CHAPTER: VI Conclusions and perspectives

There has been an increasing global interest in plastic pollution in recent years. It is seen that the scope of plastic pollution in nature is increasing at the same rate with increasing awareness and research on this pollution. In these plastic pollution studies, plastic particles found in nature are classified according to their sizes and then interpreted only on their numbers. However, the effect of plastic pollution on creatures is still not fully known, and only estimates are made. However, it is known that various additives used in plastic production are harmful to living things. Nevertheless, it is also known that various harmful materials from around the plastic particles adhere to them. Furthermore, some studies have also proven that the possible transmission of additives and adherents to creatures is related to the surface area of the plastic particle. For these reasons, it was thought that the keyword in plastic pollution was the surface area, and the point of view of the thesis was based on this value.

Within the surface area framework, it was observed that the total surface area of 7120 particles obtained at twelve different depths, which were analyzed at three stations in the water column in the Chapter II, was only equal to 70% of 1-yen. This data alone is a clue to standardize the surface area to understand the extent of plastic pollution. On the other hand, in the aforementioned chapter, has it been shown that a plastic particle with a 1-yen surface area can be divided into seven thousand particles and spread in the water column.

In the Chapter III, it has been suggested that organic structures such as marine snow are effective in the vertical transport of plastics, although this has not been proven directly and has only been hypothesized.

In the Chapter IV, plastic particles were searched in the guts of zooplankton collected in the same samples. Contrary to expectations, it was not found. However, considering the amount of plastic in the environment and the lower limits of detection. Therefore, it can be thought that the probability of its detection is not very high.

However, it is a known issue that zooplankton ingest plastic. However, the plastic encapsulating capacity of zooplankton is completely unexplored. The encapsulation issue is important for the zooplankter and its role in the ecosystem. This topic, which shows how many plastic particles can be encapsulated in the gut simultaneously, makes plastic transfer at trophic levels better understandable. Therefore, the Chapter V was built on the plastic encapsulation ca-

pacity of a zooplankton using the model zooplankton *Daphnia magna*. In the study of the Chapter V, which was performed on six different media, it was observed that *D. magna* could not differentiate between food and plastic. The fact that the number of plastics encapsulated by a single *D. magna* in the analysis results was equal to two soccer balls in surface area after four hours shows the power of the potential effect of plastic pollution on zooplankton. In addition, the method of finding the amount of plastic a *Daphnia magna* encapsulates using the direct gut volume created in this study can be applied to all other zooplankton with visible gut. Even with this novel method, the consumption amount of zooplankton fed with spherical phytoplankton species can be calculated. Thus, it can be a technique that can be used in order to bring the cost to the most appropriate values in the producers who use zooplankton species such as aquaculture by enriching them.

Perspectives on zooplankton microplastic studies,

In Chapter IV, it was stated that plastic material was sought in the guts of zooplankton collected in the MOCNESS sampling in the Sea of Japan, but it was not found in any zooplankter gut. While mentioning the reasons for this situation in the discussion section, some of the gut content may have been lost due to the possibility of stressing the zooplankton during the sampling or relaxation of the anus muscles when fixed. For example, in Chapter V, after the plastic feeding experiment, it was noticed that *Daphnia magna* left their gut content after fixation with 3% formalin solution. As an example of this relaxation of the anus and the removal of gut contents from the body, the photographs of *D. magna* were recorded in two different nutrient media (*Chlorella vulgaris* and microplastic and only in microplastic media) are displayed in Figure 6.1.1.

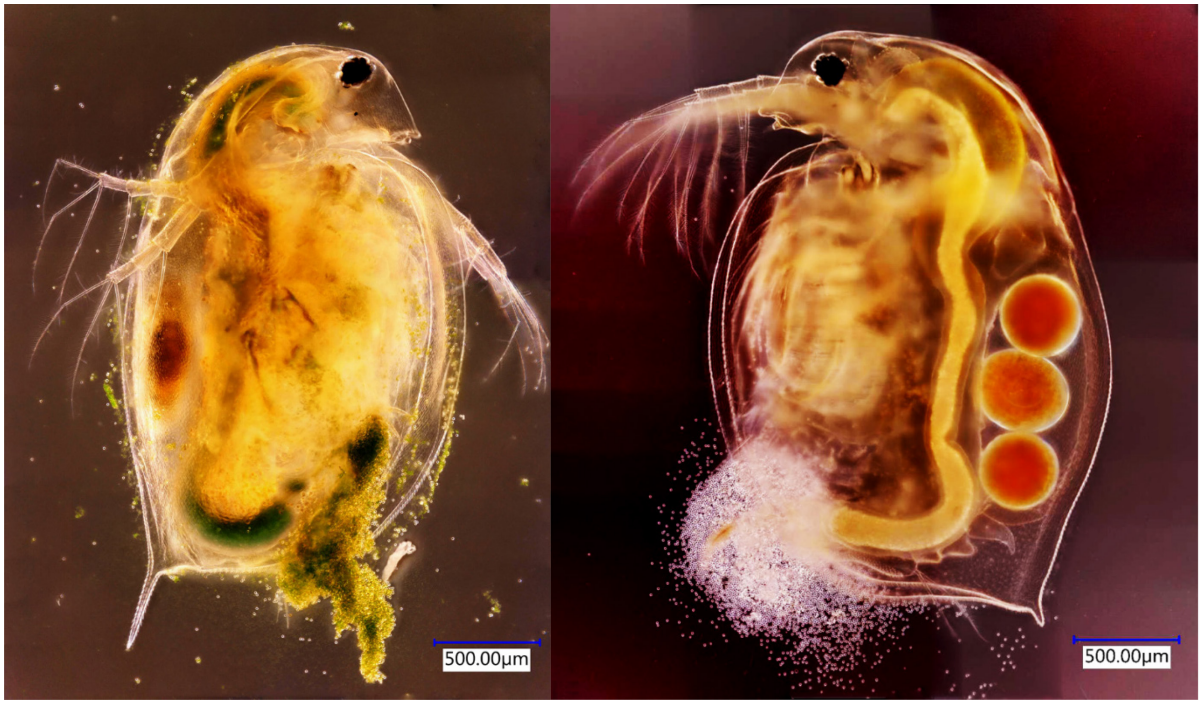

Figure 6.1.1. The photographs of the relaxation of the anus muscles and the expulsion of the gut contents after the formalin fixation of *Daphnia magna*. The *D. magna* in the left photograph was kept in a nutrient medium containing *Chlorella vulgaris* and microplastics. The one on the right was kept only in a microplastic environment.

Therefore, being able to detect the gut contents of zooplankton in their habitat may be a major scientific step. For this, the marriage of two existing devices may be a solution. For example, plastic-contaminated zooplankton should focus on a technology that can marry the µ-FTIR system with field-usable instruments such as Underwater Vision Profiler (hereinafter UVP). Zooplankter gut content can be detected instantly *in situ* with such an instrument. As of now, although it is not possible to make such a composite machine, I think that it is not theoretically impossible. Although, as of the moment, UVP photo quality cannot give a clear image of gut contents as much as a digital camera, it is not hard to believe that it will be possible with technological progress. Today, the image quality of UVPs is sufficient to determine the taxonomic groups of zooplankton. For example, the photographs obtained in the UVP sampling applied at the same cruise are shown in Figure 6.1.2. In addition, with such a composite machine, gut content can be detected not only in crustacean zooplankton, but also in gelatinous zooplankton damaged by the net.

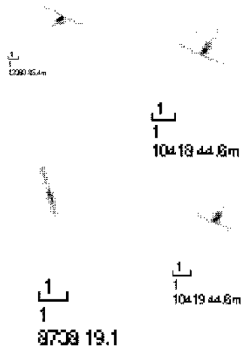

Figure 6.1.2. The photographs of zooplankton were collected from the Underwater Vision Profiler operation from the Sea of Japan.

Perspectives on plastic toxicity,

In Chapter I, the chemicals used in plastic production and their reasons were explained. Some of these chemicals are used to change the structure of plastics. On the other hand, heavy metals are added only to dye plastics. These heavy metal types vary in color and have severely harmful effects on creatures. My perspective on plastic is that we cannot prevent chemicals from the environment from sticking to the plastic, but we can limit the additives used in plastic production. For this issue, of course, additives that provide structural strengthening cannot be prevented, but additives added for cosmetics can be limited.

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