



Università
Ca' Foscari
Venezia

Master's Degree
in Environmental
Sciences
Thesis

**Assessment of the ingestion of aged microplastics by
mussels in an exposure experiments**

Supervisor

Prof. Andrea Gambaro

Dr. Fabiana Corami

Co-supervisor

Dr. Beatrice Rosso

Graduand

Sevilia Aetdinova

893010

Academic Year

2023/2024

Table of contents

| | |
|--|----|
| Abstract..... | 3 |
| List of abbreviations..... | 4 |
| 1 Introduction..... | 5 |
| 1.1 Plastic usage and emissions..... | 5 |
| 1.2 Microplastics..... | 8 |
| 1.3 Microplastics in mussels..... | 11 |
| 1.4 Goals of the thesis..... | 14 |
| 2 Materials and methods..... | 15 |
| 2.1 Pretreatment (oleoextraction, purification and filtration) of mussels and seawater from the exposure experiment..... | 15 |
| 2.2 Analysis of microplastic in Micro-FTIR Nicolet™ iN10 Infrared Microscope..... | 19 |
| 3 Results and discussion..... | 20 |
| 3.1 Microplastics in mussels in the control and the two exposition tanks..... | 20 |
| 3.2 Microplastics in seawater from the different mesocosms..... | 24 |
| 3.3 Comparison between the first and the second mesocosms..... | 33 |
| 3.4 Comparison of water and mussels from the first mesocosm..... | 36 |
| 3.5 Comparison of water and mussels from the second mesocosm..... | 38 |
| 4 Conclusions..... | 40 |
| 5 | |
| References..... | 41 |

Abstract

Microplastics are synthetic solid particles or polymeric matrices, they cannot be dissolved in water, their size limits vary, the shape is regular or irregular. Large-scale plastic production and usage started around 1950 and increased during 60 years. Plastic is used in different spheres of life.

Microplastics have been observed in different animals: birds, mammals, mussels and others. Mussels can be a bioindicator of microplastic pollution. Microplastic particles may lead to significant immunological impacts, have higher potential for mortality and have toxic chemicals.

The objective of this research was to check whether aged microplastics may be ingested by mussels. An experiment of exposure was set up and aged microplastics were produced on purpose for this experiment. The activities of this study were the quantification and identification of aged microplastics in mussels and marine waters in mesocosms.

According to the results, it cannot be established that aged microplastics were ingested more than the other microplastics, since there are environmental microplastics, including polyester, in the seawater of the controls.

Size is the principal driver for the ingestion; hence, aged microplastics may have not been of the suitable size for ingestion and they were excreted with pseudofeces. Besides, the resuspension of polyester particles may have not been efficient and those aged particles may have been at the bottom of the tank and they were not completely recovered when collecting water at the start and at the end of the exposure experiment.

Future studies are necessary to set up some crucial variables such as the size of the aged particles, the resuspension of these particles inside the tank for the whole experiment, the quality assurance/quality control protocols to check and minimize the plastic contamination constantly throughout the experiment, the necessity to improve the quality of the seawater filtration and to cover the tanks better throughout the exposure experiments. Besides, the duration of the exposure should be increased to observe and effective ingestion by the mussels.

List of abbreviations

EAA - ethylene-acrylic acid copolymer salt,
ECTFE - ethylene chlorotrifluoroethylene,
EMA - ethylene-methyl acrylate copolymer,
EVA - ethylene-vinyl acetate,
EVOH - ethylene vinyl alcohol,
HDPE - high-density polyethylene,
LDPE - low-density polyethylene,
PA - polyamide,
PARA - polyacrylamide,
PA6 - polyamide 6,
PC/PET - polycarbonate/polyethylene terephthalate,
PP - polypropylene,
PTFE - polytetrafluoroethylene,
PE - polyethylene,
PEA - polyetheramine,
PEI - polyetherimide,
PES - polyester,
PMA copolymer - polymethacrylate copolymer,
PMMA - poly(methyl methacrylate),
PO - polyolefin,
POM - polyoxymethylene,
PP + EDPM - polypropylene + ethylene propylene diene monomer,
PPA - polyphthalamide,
PPE - polyphenylene ether,
PS - polystyrene,
PU - polyurethane,
PVA - polyvinyl alcohol,
PVDC - polyvinylidene chloride,
PVDF - polyvinylidene fluoride,
SBR - styrene-butadiene rubber,
TFE-PPVE - tetrafluoroethylene-perfluoro(propyl vinyl ether) copolymer,
VE - vinyl ester.

1 Introduction

1.1 Plastic usage and emissions

In the 21st century, people produce more plastic than before. Large-scale plastic production and usage started around 1950 (Geyer et al., 2017). Production increased more than 65 times during 60 years (figure 1).

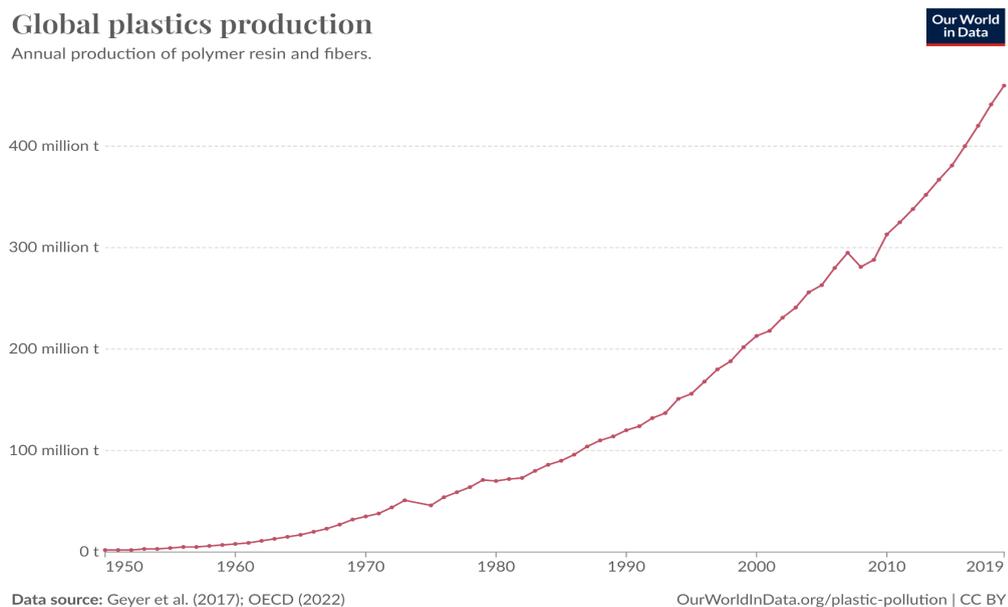


Figure 1 - Global plastics production. Source: Our World in Data, Plastic Pollution Data, <https://ourworldindata.org/plastic-pollution>

Nowadays, plastic is used everywhere; we use it in different spheres of our life: in the production of toothpastes (Carr et al., 2016), bottles, window frames, blood bags, food jars, cups, plates, food containers, rubbish bags etc (Peñalver et al., 2020). The chemical composition of microplastics in the environment is highly variable, even though 92% of the plastics produced are made up of polyethylene, polypropylene, polyvinyl chloride, polyethylene terephthalate, polyurethane, polystyrene and polyester (Geyer et al., 2017). There is a difference between the amount of different plastic elements in Asian and European continents. There is more polystyrene, polyester, polyethylene, and polyvinyl chloride in Asia than in Europe. However, the most common types of microplastic are polypropylene and

polyethylene in both parts of the world (figure 2). The time of using plastic is also different. Plastic for packaging is usually used in the year of production, while construction plastics have a longer lifespan (Geyer et al., 2017).

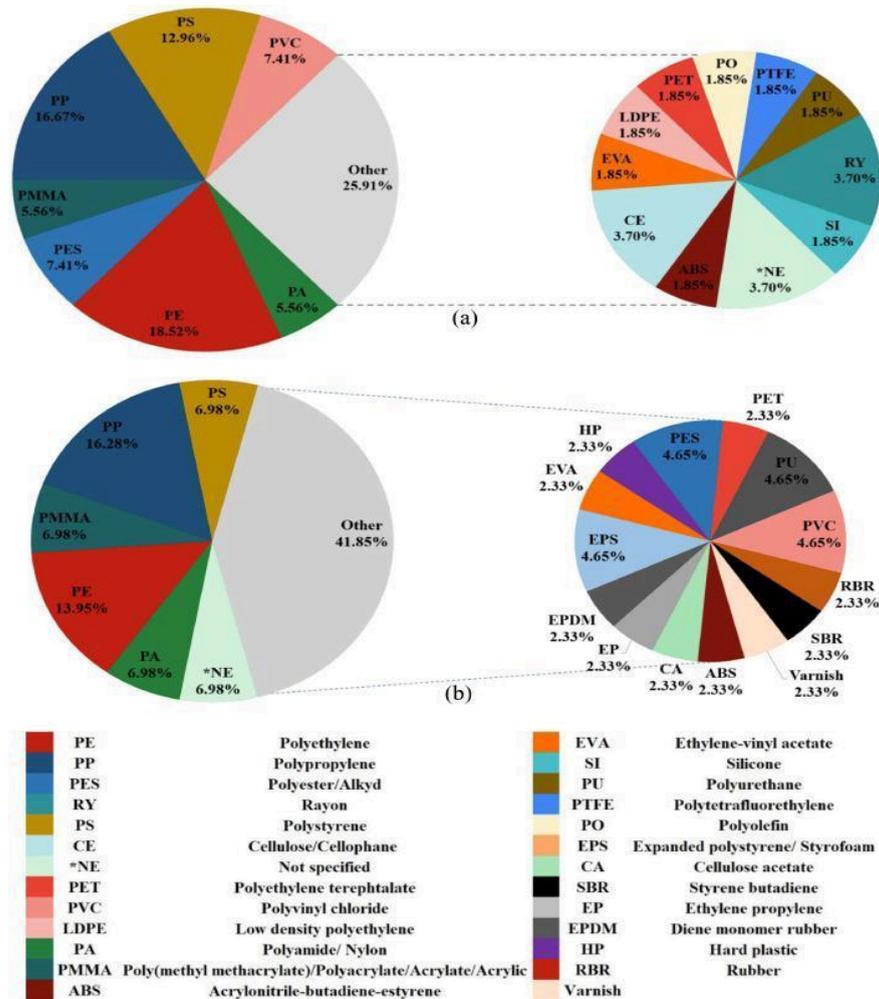


Figure 2 - Different types of microplastics in Asian (a) and European (b) continents. Source: Rossatto et al. 2023

There was about 6300 Mt of plastic waste in 2015 (Geyer et al., 2017). Widely used plastics are not decomposable, so 79% of plastic garbage has been collected in dumps or not correctly disposed of in the environment. There will be about 12000 Mt of plastic litter there in 2050; 50% of it will be incinerated, 44% will be recycled (Geyer et al., 2017).

Plastic emissions are not equal in different parts of the world. There are more plastic emissions in India, China and Brazil than in Russia and Australia (figure 3). Besides, the amount of microplastics in the Northern hemisphere exceeds its amount in the Southern (Eriksen et al., 2014, Barnes et al., 2009). Marine plastic pollution is widespread worldwide and irreversible, so satisfies two of the three suggested essential conditions for establishing a planetary boundary for chemical pollution (Villarrubia-Gómez et al., 2018).

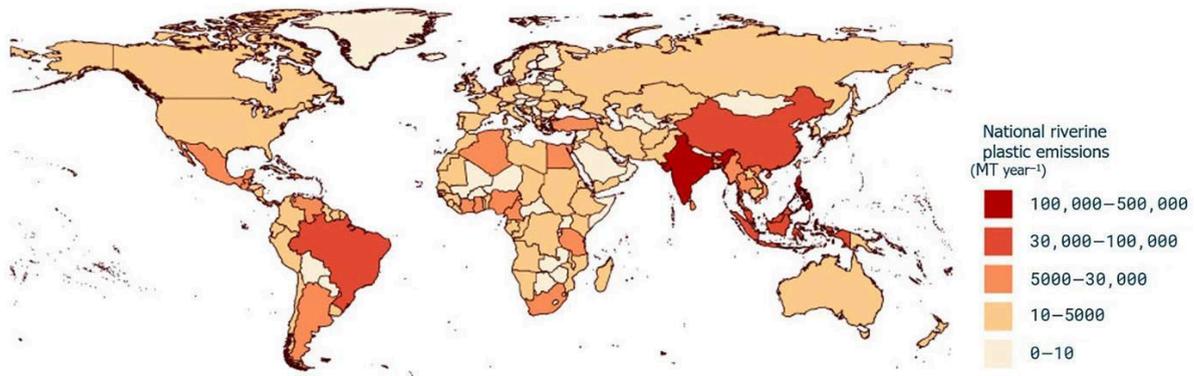


Figure 3 - Total emitted plastic into the ocean ME (MT year⁻¹) per country (Meijer et al., 2021)

1.2 Microplastics

One of the definitions of microplastics is that they are synthetic solid particle or polymeric matrix, it cannot be dissolved in water, its size limits varies: lower size limit fluctuates from 1 to 20 μm , upper size limits - from 500 μm to 5 mm, the shape is regular or irregular (Frias et al., 2019). However, the most complete definitions of microplastics has been given by the European Chemical Agency in 2019: microplastic is a material composed of solid polymer-containing particles, to which additives or other substances may have been added, with particle dimensions ranging from 1 nm to 5 mm, fiber lengths ranging from 3 nm to 15 mm, and a length-to-diameter ratio of >3 (ECHA report, 2019).

Microplastic can be primary or secondary. Primary microplastics are produced with a microscopic size, they are used in medicine, cosmetics, facial-cleansers and other items (Cole et al., 2011). They break down to secondary microplastics, which then can continue degradation because of physicochemical and biotic factors (Peñalver et al., 2020) and via surface ablation (Andrady et al., 2017) or ultrasound treatment (von der Esch et al., 2020).

The amount of microplastic particles increases over time, while their size decreases (figure 4).

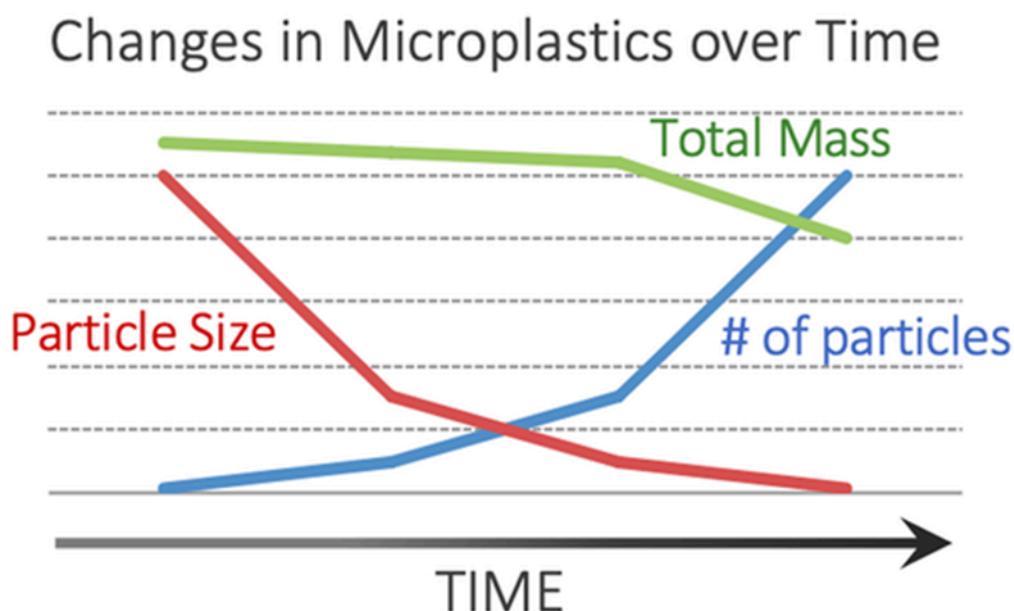


Figure 4 - Changes in microplastics over time (Hale et al., 2020)

In some cases primary microplastics are less toxic than secondary (Xia et al., 2022). According to the International Union for Conservation of Nature, the environment receives a higher amount of primary microplastics than secondary ones (Boucher et al., 2017). Secondary microplastics, unlike primary microplastics, can be eliminated from nature before they degrade (Rheinberger et al., 2021).

In autumn 2023 new regulation of the European Commission was published in the Official Journal of the European Union (<https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32023R2055>). This regulation prohibited selling of synthetic polymer microplastics as individual substances or as components of products that include them. There are biodegradable and conventional microplastics. Biodegradable microplastics are more prone to ageing and can be the source of more microplastic particles, toxic chemicals more common and more effective in this type of microplastic (Bao et al., 2020).

Microplastics are polymers (Rohrbach et al., 2023). The characteristics of a polymer can change due to high temperatures, it transits from a glassy state to a rubbery state (Corami et al., 2020b). So, microplastics' features may be affected by elevated temperatures.

There are microplastics in rivers (Devereux et al., 2022, Kelleher et al., 2023), lakes (Malla-Pradhan et al., 2022, Baldwin et al., 2020), seas (Nematollahi et al., 2020), estuaries (Laursen et al., 2023), creeks (Rimondi et al., 2022) and ocean (Boucher et al., 2017). However, plastic contamination is not equal in different places. There is a lot of macroplastics and microplastics in freshwater systems (Meijer et al., 2021, Li et al., 2018). Plastic accumulates in subtropical gyres (Eriksen et al., 2014). Microplastics were found in the ocean. In 2014 there were at least 5.25 trillion plastic particles weighing 268,940 tons in the global ocean, 75.4% of which were macroplastics, 11.4% were mesoplastics, and 10.6% and 2.6% were two microplastic size classes (Eriksen et al., 2014). However, microplastics can be efficiently eliminated by current wastewater treatment methods (Carr et al., 2016), so, it is essential to use them all over the world to decrease the amount of microplastics.

Different factors influence plastic distribution, for example, wind and waves (Iannilli et al., 2020, Imhof et al., 2018). There is a positive correlation between human activities and microplastic contamination (Rossatto et al., 2023). Flooding decreases the amount of microplastic in river beds (Hurley et al., 2018). The amount of plastic produced correlates positively with the amount of carbon dioxide (Oktavilia et al., 2020). Population and land use influence microplastic type, but not microplastics concentration, which has positive correlation with the size of sediment clays (He et al., 2020). UV rays cause degradation of microplastics, which is also temperature dependent (Andrady, 2015). Such degradation may also be caused by surface ablation and frictional forces (Andrady, 2017).

The ecological impact of microplastics is influenced by their characteristics (Andrady, 2017). However, they are dangerous for health, because they are capable of consuming pollutants, hazardous compounds (Cole et al., 2011, Crawford et al., 2017) and toxins (Li et al., 2018). Bacterial pathogens, transported by microplastic, are the reason for coral albinism (Junaid et al., 2022). Floating plastic can also transport some components of flora and fauna (Eriksen et al., 2014).

As a result, food chains change and disrupt (Junaid et al., 2022). Besides, microplastics are colonised by microorganisms (Zettler et al., 2013, McCormick et al., 2014), including pathogens (Kirstein et al., 2016, Junaid et al., 2022). They contain heavy metals (Cole et al., 2011). Microplastic is a cause of disturbances in feeding and energy metabolism and physiological changes in the liver (Anbumani et al., 2018). It results in neurodegenerative diseases, oxidative stress, deterioration of immunity and cytotoxicity. Microplastics can penetrate cell membranes, damage them and change their properties (Wang et al., 2022). They can reduce or increase the amount of energy intake in an organism and lead to negative energy balance and lower ATP amount. Microplastics can reach the blood flow, liver, kidneys, spleen, placenta and intestines, and can affect neuronal function and behaviour. Besides, it may present chemical and biological hazards (Prata et al., 2020). Fish behavioural responses are changed by microplastics (Barboza et al., 2018).

1.3 Microplastics in mussels

Microplastics have been observed in different animals: birds (Wayman et al., 2024, Tokunaga et al., 2023), mammals (Katlam et al., 2022, Li et al., 2023), fishes (Horton et al., 2024, Srisiri et al., 2024), in oysters (Corami et al., 2020), in amphipods (Iannilli et al., 2019) and also in mussels (Jong et al., 2022).

There is a positive correlation between the amount of microplastic in animals and microplastic particles in their environment (Iannilli et al., 2020). Besides, there is a strong connection between the amount of microplastics in mussels and in the reservoir in which they live (Qu et al., 2018). The rate of microplastic fibres absorption by mussels rises with increasing microplastic fibres concentration and microplastic accumulation increases with time (Woods et al., 2018), so mussels can be used to detect microplastic contamination and assess the scale of pollution.

Mussels can be a bioindicator of microplastic pollution in shoreline ecosystems and potentially they can be employed in bioremediation, because they may contribute to biological purification (Patterson et al., 2021). In the research of Patterson et al., 2021 blue microplastic particles and polyethylene predominated among other types. So it is possible to check, using mussels, if in other water bodies the same types of microplastic predominate or not.

There are more small than large microplastic particles in mussels (Qu et al., 2018, Kolandhasamy et al., 2018). Salinity of water can also influence the size of microplastic in mussels: microplastic particles from low salinity and brackish water were smaller than in high salinity sea water (Khoironi et al., 2018). On one side, there is a connection between size and toxicity, small particles are more toxic for mussels and lead to significant immunological impacts (Jong et al., 2022). On the other side, large microplastics have higher potential for mortality. Different types of plastics have different toxicity: polystyrene is more toxic than polypropylene, which is more toxic than polybutylene succinate (Phothakwanpracha et al., 2021). Polybutylene succinate is a biodegradable plastic. However, in general biodegradable microplastics are more likely to have toxic chemicals, which are more effective in this type of microplastic (Bao et al., 2020). But bio-based and biodegradable microplastics are not toxic for mussels (Khalid et al., 2021).

Mussels of different sizes have different amounts of microplastic particles. Big mussels absorb microplastic slower and ingest less microplastic per gram of tissue than small mussels (Patterson et al., 2021). However, microplastic influences mussels of any size. Consumed microplastics get into the mussel's circulatory system (Browne et al., 2008) and into digestive tissues (Aramendia et al., 2024). The presence of microplastic fibres can reduce filtration rate by approximately 2 times (Woods et al., 2018).

The largest amount of microplastics was found in the intestine of mussels (Kolandhasamy et al., 2018). They were most likely swallowed because the mussels mistook them for food. However, in

addition to standard methods of absorbing microplastics, such as during breathing and feeding, mussels have another one. They can absorb microplastics during adherence; because of this, it can accumulate in organs of the body wherever it ends up after ingestion, for example, foot (Kolandhasamy et al., 2018). In this way, a significant proportion of microplastics enter the mussels, 42–59% of the total amount of microplastics in their body. Besides, there are microplastics in the digestive gland and gills (Woods et al., 2018). 77% of microplastics in mussels are fibres, probably because of slower expulsion of fibres and because most microplastics in a water body are fibre (Patterson et al., 2021). Length of ingested microplastic fibres varies significantly in different parts of mussels' bodies: the longest have been found in the gill, shorter ones have been found in the digestive gland and the shortest have been found in soft tissue (Woods et al., 2018). There is a connection between the accumulation of microplastics in different organs and its concentration in a body of water. At high microplastic fibres concentrations, more than 80% accumulates in the digestive gland, at middle concentration there are less than 50% of microplastic fibres in this organ (Woods et al., 2018). In addition to accumulation, mussels also remove some of the accumulated microplastic over time: in microplastic-free water they can remove about 63% after 6 hours, at the same time microplastic removes faster from the gill than from digestive gland (Woods et al., 2018). Microplastic fibres stay in the gut for a minimum of 3 days.

There is a connection between human activity and the amount of microplastic in mussels; besides, mussels accumulate microplastics effectively: there are more small microplastics in proportion than environmental samples in the bodies of mussels (Patterson et al., 2021). The rate of microplastic fibres absorption by mussels rises with increasing microplastic fibres concentration and microplastic accumulation increases with time (Woods et al., 2018). Mussels are bioindicators, it is possible to assess the degree of microplastic contamination, using this type of animal (Patterson et al., 2021, Li et al., 2019).

Besides, studying microplastic particles in mussels, it is possible to understand how they interact with biota, water studies do not provide such an opportunity (Bråte et al., 2018). For example, in mussels the presence of microplastic fibres can reduce filtration rate by approximately 2 times (Woods et al., 2018).

Mussels are a source of food for human beings. It is necessary to control mussels' quality to maintain a high level of people's health, because mussels can contain something harmful. Several dangerous substances were found in mussels. Bisphenol A, which is an endocrine disruptor, was found in Norwegian mussels (Bråte et al., 2018). Also, pesticides, perfluoroalkyl substances, pharmaceuticals and personal care products were found in mussels (Álvarez-Ruiz et al., 2021). Besides, when people eat mussels, they become exposed to microplastics and other substances that microplastics contain. Microplastic is harmful for health, it causes neurodegenerative diseases,

physiological changes in the liver, penetrate and damage cell membranes (Anbumani et al., 2018, Wang et al., 2022). It is essential to study microplastics entering us through food.

Microplastics in mussels may contain chemical elements, such as Ca, Mg, K, Sr, Cl, pollutants, iron oxides and hydrophobic chemicals, resisting decomposition (Patterson et al., 2021). Microplastics can also include additives such as bisphenols and phthalates, the amount of which can be reduced in mussels because of water and/oil (Gamarro et al., 2024). So it is possible to reduce the concentration of these additives in mussels during cooking and it is better not to use oil and water, which were used for cooking. It will increase mussels' quality in food. Besides, bisphenol A, pesticides, perfluoroalkyl substances, pharmaceuticals and personal care products were found in mussels (Bråte et al., 2018, Álvarez-Ruiz et al., 2021).

Small microplastics seems to be more toxic for mussels (Jong et al., 2022), but large microplastics have higher potential for mortality (Phothakwanpracha et al., 2021). In Kolandhasamy et al., 2018 and Qu et al., 2018 researches mussels absorbed more small than big microplastics. Also, it is not clear which type of microplastic mussels prefer. Study of Woods et al., 2018 finds 71% of microplastic particles are rejected by them as pseudofeces. In this case it is essential to understand which type of microplastics they reject to increase the amount of potentially rejected microplastic. More research is needed on microplastics in mussels.

1.4 Goals of the thesis

The objective of this research is to check whether aged microplastics may be ingested by mussels, since microplastics in the environment have undergone fragmentation, erosion and photooxidation processes.

An experiment of exposure was set up and aged microplastics were produced on purpose for this experiment. The activities of this study, which became the focus of my thesis, were the quantification and identification of aged microplastics in mussels and marine waters in mesocosms.

This thesis work was carried out at the CNR-ISP Institute of Polar Sciences in Venice. CNR-ISP participated in a project in partnership with the University of Padua (contact person and partner Professor Gabriella Marin) and the Istituto Zooprofilattico delle Venezie (Principal Investigator. Dr. Carmen Losasso).

2 Materials and methods

The aged microplastics (polyester) were produced by Istituto Zooprofilattico delle Venezie at their laboratory in Legnaro (Padova) These particles were then added to the tanks (control and exposure mesocosms) at the Hydrobiological Station at Chioggia. The concentration in weight of these particles were in excess to the environmental concentration observed in seawater from the literature. All the variables of the exposure experiments were set and controlled throughout the experiment by the University of Padua.

Samples of mussels and seawater were collected at the start and at the end of the experiment (T0 and T7) in order to quantify and identify the microplastics using the Micro-FTIR. Mussels were frozen and seawater was stored at 4 °C and transported to the laboratory of the Institute of CNR-ISP and Ca' Foscari university for the analysis.

2.1 Pretreatment (oleoextraction, purification and filtration) of mussels and seawater from the exposure experiment

In general, there are two steps in microplastics' analysis. The first step is the pretreatment (oleoextraction, purification, and filtration) and, the second step is quantification and simultaneous identification via Micro-FTIR.

Methodology, based on the methods described by Corami et al., 2021, was used for this study.

It is important to prevent plastic contamination, otherwise research results will be unreliable, microplastic will influence them. It will be almost impossible to understand which type of microplastic and in which amount was extracted from the laboratory sample and which microplastic was part of the environment. So, the laboratory environment for all steps of research should be plastic free.

2.1.1 Sampling of marine waters from the exposure experiments

Marine waters were collected in the Hydrobiological Station of Chioggia (University of Padova) neighborhood by project members of the Department of Biology, University of Padua. Before being poured into the tanks (two for exposure and one for control), seawater was filtered to remove the particulate. Homemade microplastics from Istituto Zooprofilattico delle Venezie were aged in a tank at the Hydrobiological Station of Chioggia (University of Padova), according to a protocol, developed by Istituto Zooprofilattico delle Venezie (natural sunlight in a tank in which the particles

are always kept in suspension for about four months). The aged microplastics (polyester) were then added into the two tanks for exposure, together with mussels. Seawater from the three tanks (exposure 1 and exposure 2, control) was collected at the beginning and end of the exposure experiments and then stored in decontaminated glass bottles at 4 °C before being transported to CNR-ISP laboratories, where they would be pretreated and analyzed.

2.1.2 Quality control of analyses

Decontamination, extraction and filtration were carried out in a plastic-free cleanroom (figure 4) at the Department of Environmental Sciences, Informatics and Statistics of Ca' Foscari University and at the Institute of Polar Sciences of National Research Council (CNR-ISP) in Venezia-Mestre to avoid plastic contamination. There is regulation of temperature, humidity, atmospheric pressure and particle pollution in this laboratory (Corami et al., 2021). During laboratory experiments cotton lab coats were used.

2.1.3 Washing and decontamination

The aim of washing was removing chemicals and microplastics from last experiments. Ultrapure water was used to prevent microplastics from entering.

The glassware was washed with a 1-2% Citranox or Contrad soap solution. Then it was rinsed with warm ultrapure water and with a 50% solution of ethanol-methanol. The glassware dried under the fume hood, covered with decontaminated aluminum foil.

The glassware was rinsed with warm ultrapure water. It was filled with a prepared solution of NaOH 0,1 mol/l. The next day the glass was rinsed with warm ultrapure water and with ultrapure water at room temperature and washed with the 1-2% Citranox or Contrad soap solution. The glassware was rinsed 2-3 times with ultrapure water. Then it was rinsed with a 50% ethanol-methanol solution under the decontaminated fume hood. The glassware dried under the fume hood, covered with decontaminated aluminum foil.

The glassware was rinsed with 50% ethanol-methanol solution, with 70% ethanol-methanol solution and with ethanol for decontamination.



Figure 5 – Plastic-Free Clean Room

2.1.4 Oleoextraction of the seawater in the mesocosms

The procedure was performed according to Corami et al., 2021, with minor changes. 200 ml of well mixed seawater from sample or blank, 200 ml of ultrapure water, 5 ml of oil and 10 ml of hydrogen peroxide were poured into the first separatory funnel. These substances were mixed by shaking to form emulsion. After 14-24 hours ultrapure water was poured till the edge while the tap was closed. For recovering oil it was recovered by pouring from the top into the second funnel until two phases were formed. The water in the first funnel was poured from the top into the third decontaminated

funnel. Two phases were formed after about 30-60 minutes. Tap in the second funnel was opened to power water from the second funnel into the third funnel. Mix of 10 ml hexane and 10 ml of ethanol was poured to the second funnel to recover oil. Then 10 ml hexane and 10 ml ethanol were poured to the funnel again. 5 ml of oil and 5 ml of hydrogen peroxide were added to the third separatory funnel with the mix water to recover the oil phase. After 3 hours a check was made to see if the oil phase was clean (if clean phase is dirty, last steps should be repeated). Tap was open to pour water into a beaker. Mix of 10 ml hexane and 10 ml of ethanol was added to recover oil. The funnel was rinsed with 10 ml of hexane and 10 ml of ethanol to completely recover the oil phase. The flask was capped with double aluminum foil until filtration.

2.1.5 Sampling and pseudodigestion of mussels for exposure experiments

Mussels were collected in the Hydrobiological Station of Chioggia (University of Padova) neighborhood by project members of the Departments of Biology, University of Padua. The organisms were conditioned and collected at time 0. They were then displayed in two different exposure tanks, containing the aged plastics and collected at the end of the experiment. Besides, there was a third tank, namely the control tank, where mussels were not exposed. At the end of the exposure experiment, organisms were collected as well.

All the specimens were frozen at -20° and transported to CNR-ISP laboratories, where they were then analyzed for the quantification and identification of microplastics, according to the method developed by Corami et al., 2020, which consists of a pseudodigestion.

Organisms were pseudodigested as a whole in order to evaluate the load and the potential impact on the trophic web and human health.

Organisms were shelled and put in a decontaminated glass bottle, where hydrogen peroxide, a solution of 50% ethanol and isopropanol, and ultrapure water were added (ratio 1: 2.5: 3.2 to reach a volume of 200 ml). After the pseudodigestion (2 h at 30°C , 96 h on an orbital shaker, 140 rpm), samples were diluted, filtered, and purified according to Corami et al. (2020). The filters employed were Anodisc filters (Whatman, $0.2\ \mu\text{m}$, 47 mm). After filtration and purification, all filters were stored in decontaminated Petri dishes, covered with aluminum foil, and dried for 72 hours at room temperature under a laminar flow hood.

2.1.6 Filtration and purification

Anodisc filters are thin aluminum filters that have a honeycomb structure that resists deformation (Corami et al., 2020a). These filters were used in the filtration step.

A filter was flushed with 10 ml of 70% ethanol-methanol twice, with ethanol once. Part of the oleo extract solution was filtered. The filter was flushed with 5 ml of hexane and with an aliquot of 70% ethanol-methanol. Another part of the oleo extract solution was filtered. The filter was flushed with 70% ethanol-methanol solution and with ethanol. The last part of the oleo extract solution was filtered. The filter was flushed with 70% ethanol-methanol solution and with ethanol several times. All replicates of a sample were filtered, using one filter for one sample. The filtering system was thoroughly decontaminated before filtering another sample. Filters were put in decontaminated Petri dishes, which were left slightly opened, under fume hood to let the filter dry before analysis.

2.2 Analysis of microplastic in Micro-FTIR Nicolet™ iN10 Infrared Microscope

There are different methods for microplastic quantification and qualification. Liquid chromatography, Raman spectroscopy, FTIR spectroscopy and pyrolysis-GC/MS are used for microplastics qualification (Li et al., 2018).

Analytical techniques for microplastics investigation must unambiguously identify the polymers; these techniques are spectroscopic techniques like FTIR and Raman (Hanvey et al., 2017).

For this study, the analysis were performed via a Micro-FTIR Nicolet™ iN™ 10 Nicolet™ iN10 Infrared Microscope (Thermo Fisher Scientific), which is based on Fourier transform infrared spectroscopy (FTIR) and includes mercury cadmium telluride detector and an ultra fast-stage (Corami et al., 2020a).

Fourier transform infrared spectroscopy is a methodology utilized to acquire an infrared spectrum of absorption or emission. It is used in different analyses, for example, in pharmaceutical analysis.

The filter was put under the Micro-FTIR Nicolet™ iN™ 10 Nicolet™ iN10 Infrared Microscope (Thermo Fisher Scientific) and the Particles Wizards was employed to analyze in transmission mode each count area or count field , randomly chosen without overlapping. Operating with Particles Wizard allows to quantify the microplastics via microscopic counting and simultaneously identify the polymers. The spectra of the particles were then compared to spectra in several different reference libraries. Only the spectra of particles retrieved with an optimal match of similarity (>65%) were considered optimally identified and then quantified (Corami et al., 2021).

3 Results and discussion

3.1 Microplastics in mussels in the control and the two exposition tanks

Polymers identified and quantified in mussels included olefin, poly(ethyl acrylate), polyacrylic, PTFE, PA6, PP, PPA, PARA, modacrylic and PE (table 1). Mussels in the Control tank included more polymers than those in the two exposition tanks.

Control sample T0 included 894 SMPs/L, control sample T7 included 1950 SMPs/L. Experimental samples included 551 SMPs/L and 120 SMPs/L. So the abundance of microplastics in control samples was 4 times more than in experimental samples.

Polyacrylic and polytetrafluoroethylene were found in control samples, but they were not detected in experimental samples. In contrast, polyethylene was found only in experimental samples. Abundance of modacrylic, polyarylamide and olefin in control samples was much more than the abundance of these polymers in experimental samples: 6.67 times more for modacrylic and polyarylamide, 5.25 times more for olefin.

Poly(ethyl acrylate) was 17 SMPs/L both in experimental and control samples. Abundance of polyamide 6 and polyphthalamide was less than in experimental in the first control sample and much more than in experimental in the second control sample. In contrast, the abundance of polypropylene in the control sample was less than in the second experimental sample and more than in the first experimental sample.

Polymer composition was different in experimental samples (figures 6 and 7). There was more polyamide 6 in mussels from the first pool and more polypropylene in the second pool. Polypropylene was isolated both from mussels from the first and second pools, but other polymers were different in these pools. Polymer composition was less diverse in mussels from the second pool, there was only polypropylene, polyethylene and poly(ethyl acrylate) there. Mussels from the first pool included olefin, polyamide 6, polypropylene, polyphthalamide, polyarylamide and modacrylic.

| | Pool Control T0 | Pool Control T7 | Pool exposed Mesocosm 1 T7 | Pool Exposed Mesocosm 2 T7 |
|--|-----------------------|--------------------|-------------------------------|-------------------------------|
| olefin | 361 | | 69 | |
| poly(ethyl acrylate) | 17 | | | 17 |
| polyacrylic | 86 | | | |
| polytetrafluoroethylene | 17 | | | |
| polyamide 6 | 292 | 1262 | 327 | |
| polypropylene | 69 | | 34 | 86 |
| polyphthalamide | 52 | 344 | 69 | |
| polyarylamide | | 115 | 17 | |
| modacrylic | | 229 | 34 | |
| polyethylene | | | | 17 |
| Total for every sample (n SMPs/organism) | 894 | 1950 | 550 | 120 |
| Fiducial interval according Poisson distribution (error) | 41 | 61 | 32 | 15 |

Table 1 - Polymers observed in mussels before and after the exposition in the two mesocosm

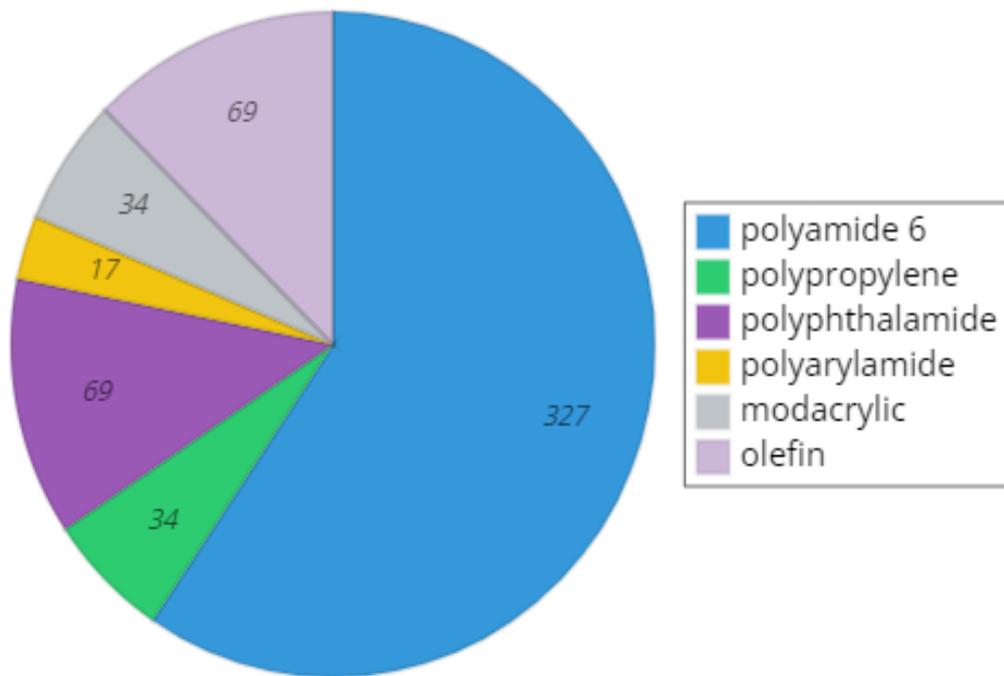


Figure 6 - Polymers in mussels from the first pool

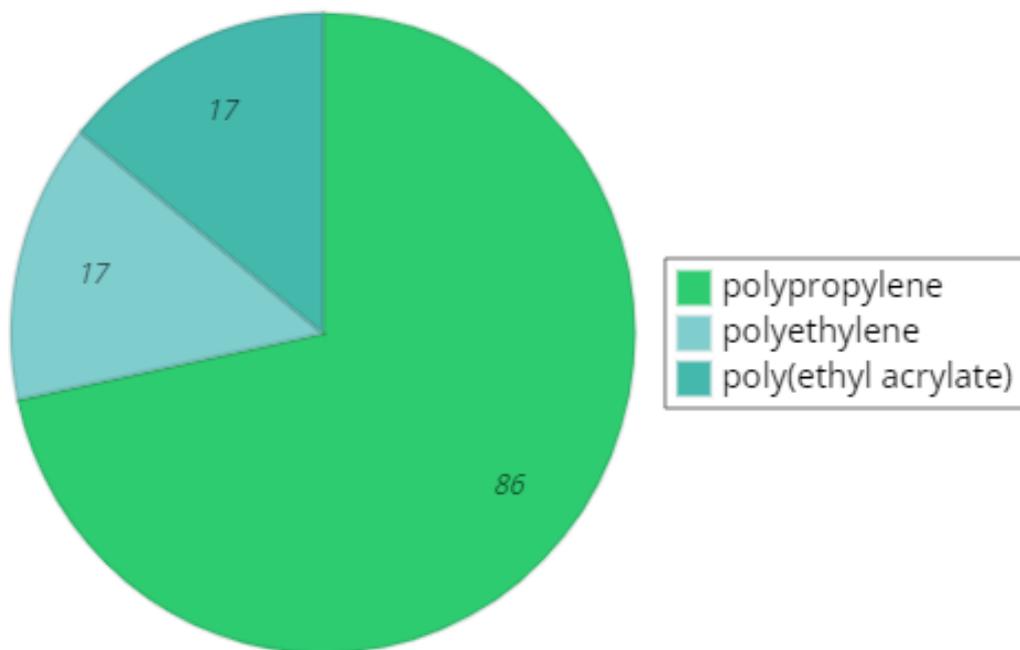


Figure 7 - Polymers in mussels from the second pool

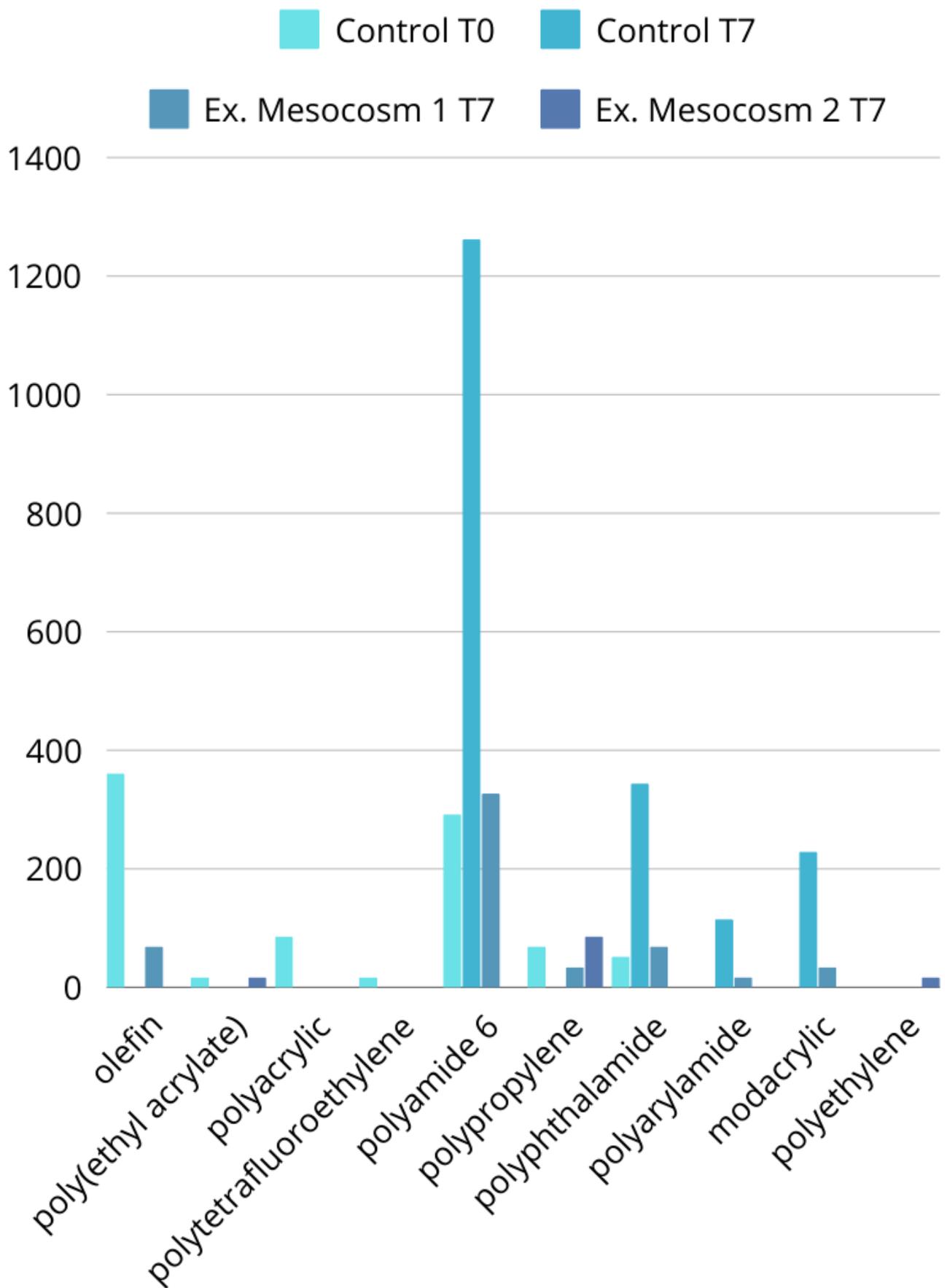


Figure 8 - Polymers observed in mussels before and after the exposition in the two mesocosm

3.2 Microplastics in seawater from the different mesocosms

3.2.1 Microplastics in the first mesocosm

List of polymers observed in the first mesocosm and in its control is in table 2. These polymers were extracted from the mesocosm of the exposure experiment and from control samples at the start of the experiment (T0) and the end of the experiment (T7). The samples were analyzed at least in duplicate, and the abundance is expressed as an average abundance with the fiducial interval according the Poisson distribution (error)

| Polymer | Exposition T0 | Exposition T7 | Control T0 | Control T7 |
|---------------------------|---------------|---------------|------------|------------|
| polyolefin | 76 | | 101 | 666 |
| olefin | | | 201 | 73 |
| poly(ethyl acrylate) | | 73 | | |
| polypropylene | | | 73 | |
| polytetrafluoroethylene | 678 | 73 | 145 | 73 |
| polyamide 6 | 992 | 578 | 1277 | 479 |
| polyamide | 308 | 362 | 101 | |
| modacrylic | | 73 | | |
| high-density polyethylene | 776 | 290 | 1763 | 362 |
| polyester | 534 | 374 | 173 | |

| | | | | |
|--|------|------|------|------|
| styrene-butadiene rubber | 76 | | 101 | 73 |
| polyurethane | | | 374 | |
| polyvinylidene chloride | | | 1807 | 1711 |
| polyetherimide | 226 | 145 | 847 | 335 |
| polystyrene | | | 73 | |
| vinyl ester | 386 | 217 | | 73 |
| ethylene-vinyl acetate | 76 | | | 335 |
| ethylene vinyl alcohol | 76 | | 619 | 240 |
| polymethacrylate copolymer | | | 201 | 190 |
| polyurethane | | | 101 | |
| polyetheramine | 3440 | 1518 | 2874 | 1852 |
| polyvinyl alcohol | | | | 95 |
| polycarbonate/polyethylene terephthalate | | | 101 | |
| ethylene-methyl acrylate copolymer | | | | 95 |

| | | | | |
|---|------|------|-------|------|
| tetrafluoroethylene-perfluoro(propyl vinyl ether) copolymer | 301 | | | |
| ethylene-acrylic acid copolymer salt | | | 101 | 95 |
| poly(methyl methacrylate) | 154 | | 301 | 168 |
| ethylene chlorotrifluoroethylene | 772 | 434 | 245 | 742 |
| Total Abundance (SMPs/L) | 8867 | 4134 | 11571 | 7652 |
| Fiducial interval (error) | 131 | 89 | 149 | 121 |

Table 2 – Polymers observed in the first mesocosm and in its control at the start of the exposure experiment (T0) and at the end of the experiment (T7)

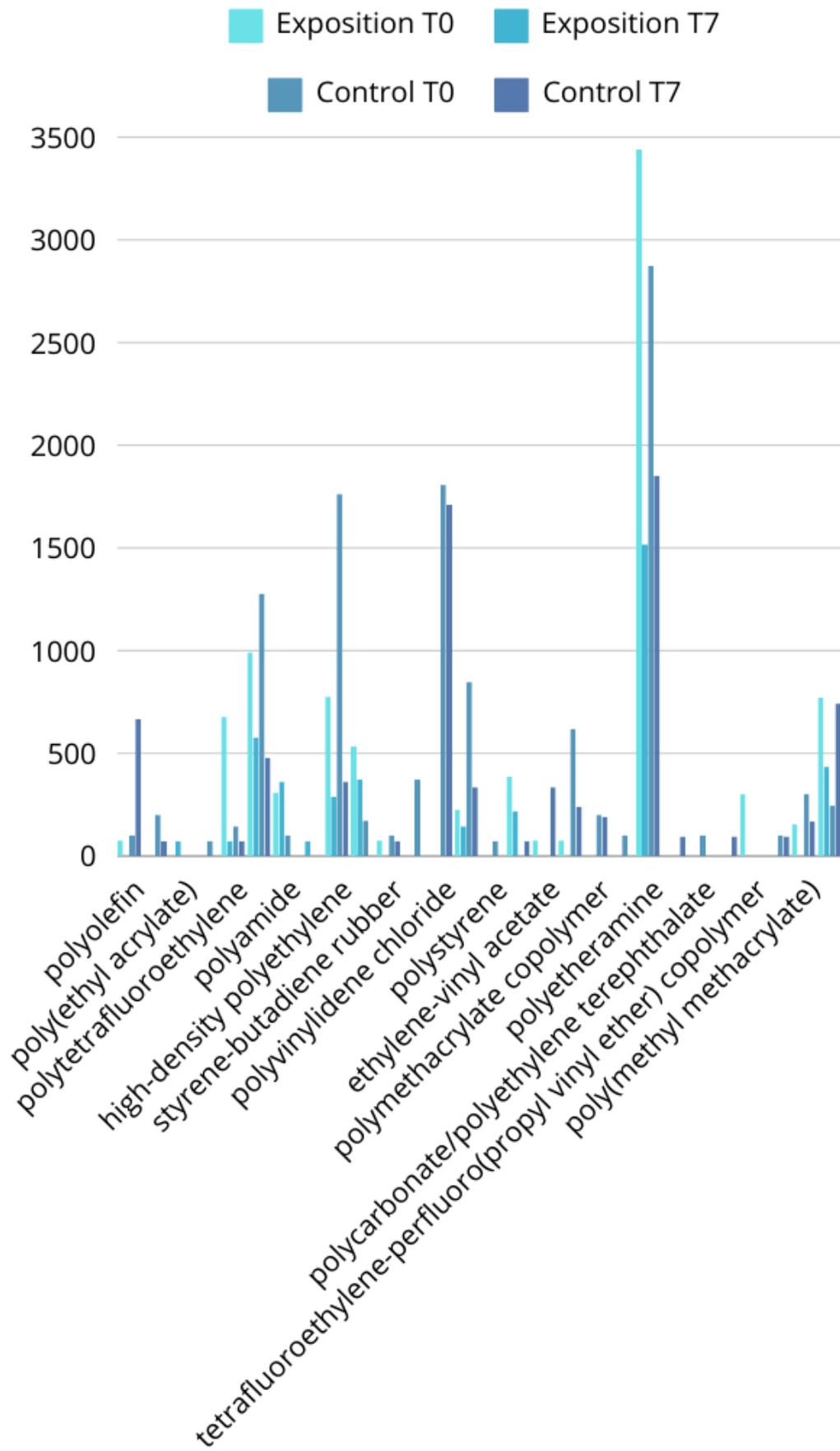


Figure 9 - Polymers, observed in the first mesocosm

Some polymers were found only in experimental samples: poly(ethyl acrylate), modacrylic and tetrafluoroethylene-perfluoro(propyl vinyl ether) copolymer. At the same time, other polymers were isolated only from control samples: olefin, PP, PU, PVA, PC/PET, EMA and EAA. Polyetheramine and ethylene chlorotrifluoroethylene were found in every sample and replica. There were 34544 SMPs/L from control samples and 25567 SMPs/L from experimental samples.

Polymer composition was different in control and experimental mesocosms. There was more poly(ethyl acrylate), PTFE, PA, modacrylic, PES, VE, PEA, TFE-PPVE and ECTFE in experimental samples. At the same time, there was more PO, olefin, PP, PA6, HDPE, SBR, PU, PVDC, PEI, PS, EVA, EVOH, PMA copolymer, PU, PVA, PC/PET, EMA, EAA and PMMA in control samples. Concentration of polyetheramine was higher than any other polymer (figure 9) in experimental mesocosms T0b and T7b.

The largest abundance of microplastics was found in the control mesocosm at the start of the experiment, while the least abundance was observed in the exposure mesocosm at the end of the experiment. The control mesocosm always has a higher abundance (SMPs/L) than the exposure mesocosm. Seawater for mesocosms was collected in the surrounding of the Hydrobiological Station at Chioggia.

Therefore, variables such as seawater filtration and its exchange and replacement in mesocosms and the resuspension of added microplastics should have been evaluated better. Besides, the plastic contamination during the exposure experiment should have been thoroughly checked, and quality assurance/quality control protocols should have been better designed so that control mesocosms should have had a lower abundance than the exposure mesocosms

3.2.3 Microplastics from the second mesocosm

List of polymers from the second pool is in table 3. These polymers were isolated from control 2 and mesocosm 2 at the start (T0) and at the end (T7) of the exposure experiment.

| Polymer | Control T0 | Control T7 | Exposition T0 | Exposition T7 |
|---|------------|------------|---------------|---------------|
| olefin | | 181 | | |
| polyolefin | | | | 91 |
| poly(ethyl acrylate) | | | | 91 |
| polypropylene | 362 | 181 | | 91 |
| polypropylene + ethylene propylene diene | | 91 | | |
| polyphenylene ether | | 91 | | |
| polyetherimide | | | 91 | |
| polyetheramine | 1626 | | 1445 | 2529 |
| polyester | 91 | 181 | 3161 | 1355 |
| polyoxymethylene | | | 91 | 91 |
| fluorocarbon | 542 | 271 | | 271 |
| polytetrafluoroethylene | 91 | 181 | 272 | |
| high-density polyethylene | 452 | 813 | 91 | 181 |
| low-density polyethylene | 91 | 181 | | |

| | | | | |
|--|------|------|------|------|
| vinyl ester | | | | 91 |
| polyvinylidene fluoride | | | | 91 |
| ethylene-vinyl acetate | | | 91 | |
| polyamide 6 | 361 | 453 | | |
| Total Abundance (SMPs/L) | 3614 | 2621 | 5240 | 4879 |
| Fiducial Interval according to Poisson distribution (error). | 83 | 71 | 100 | 97 |

Table 3 - Polymers observed in the second mesocosm and in its control at the start of the exposure experiment (T0) and at the end of the experiment (T7)

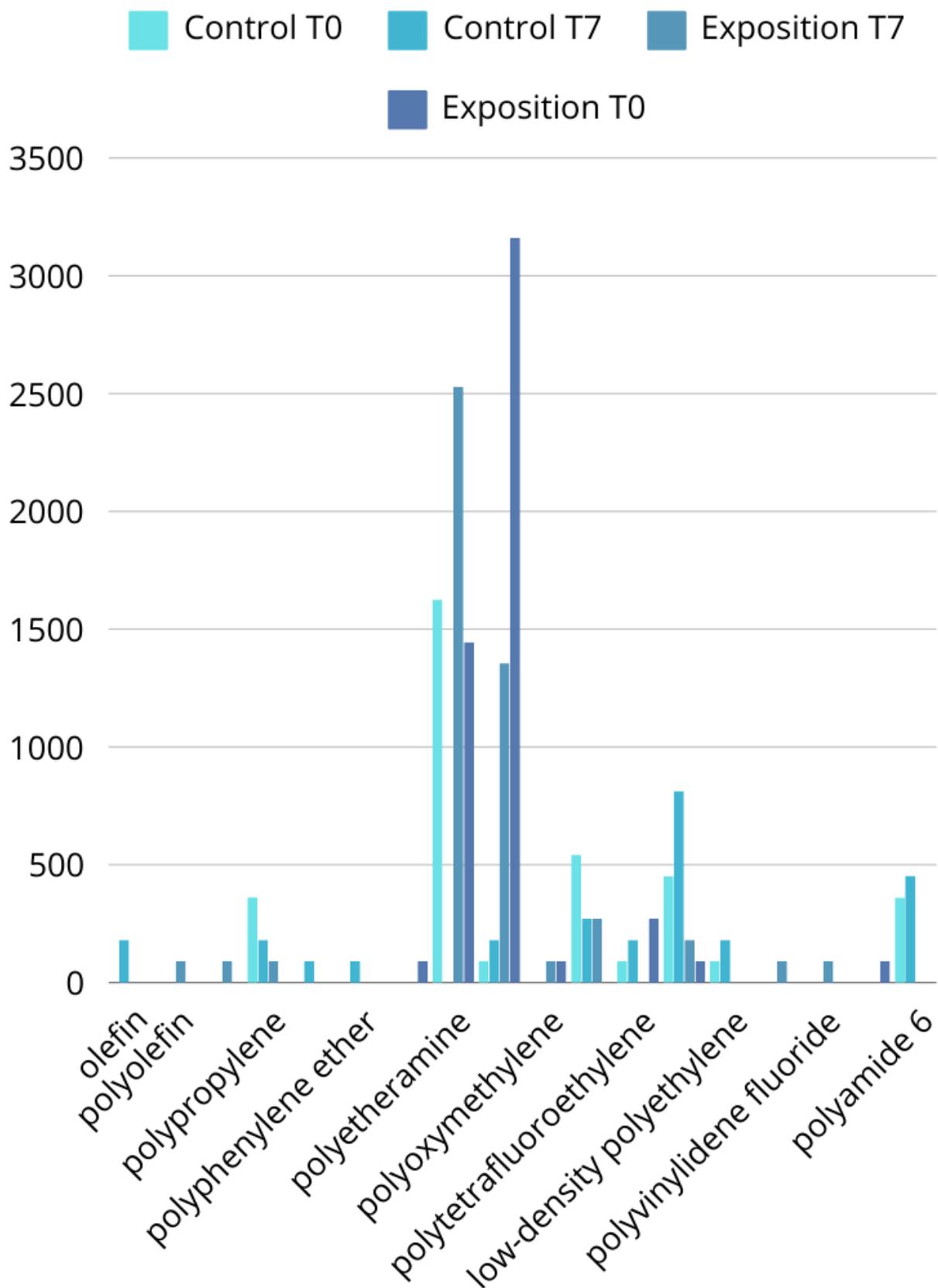


Figure 10 - Polymers, isolated from experimental samples from the second pool

Polymer composition was different in control and experimental samples. There were more PO, poly(ethyl acrylate), PEI, PEA, PES, POM, VE, PVDF, EVA in experimental samples. At the same time, there were more olefin, PP, PP + EDPM, PPE, ECTFE, HDPE, LDPE and PA6 in control samples. The abundance of polytetrafluoroethylene was almost the same in control and experimental samples. PO, poly(ethyl acrylate), PEI, POM, VE, PVDF and EVA were isolated only from experimental samples, olefin, PP + EDPM, PPE, LDPE and PA6 - only from control samples. In contrast to what was observed in the first mesocosm, the total abundances between control and exposure are similar, confirming that the failure to minimize contamination may have affected the experiment's performance.

3.3 Comparison between the first and the second mesocosms

There were 13 polymers both in the first and second pools (table 4): PO, olefin, poly(ethyl acrylate), PP, PTFE, PA6, HDPE, PES, PEI, VE, EVA, PEA, ECTFE.

Among common microplastics, the most polyetheramine was contained in both control and experimental samples in the first pool. In the second pool experimental samples contained the most abundance of polyester, control samples contained the most polyetheramine (figure 11).

Among common microplastics, in the first pool control samples contained the least abundance of polypropylene and vinyl ester, experimental samples contained the least poly(ethyl acrylate). In the second pool polyolefin, poly(ethyl acrylate), polypropylene, polyetherimide, vinyl ester and ethylene-vinyl acetate were contained in experimental samples, control samples contained the least olefin.

At the same time, there were 15 microplastic types only in the first pool: PA, modacrylic, SBR, PU, PVDC, PS, EVOH, PMA copolymer, PU, PVA, PC/PET, EMA, TFE-PPVE, EAA and PMMA. And 5 polymers were isolated only from the second pool: polypropylene + ethylene propylene diene monomer, polyphenylene ether, polyoxymethylene, low-density polyethylene and polyvinylidene fluoride. Poly(ethyl acrylate) was found only in experimental samples, olefin was isolated only from control samples both in the first and second pools.

| | Control 1st pool | Exposition 1st pool | Control 2nd pool | Exposition 2nd pool |
|---------------------------|------------------|---------------------|------------------|---------------------|
| polyolefin | 1532 | 151 | | 181 |
| olefin | 546 | | 361 | |
| poly(ethyl acrylate) | | 145 | | 181 |
| polypropylene | 145 | | 1084 | 181 |
| polytetrafluoroethylene | 434 | 1501 | 542 | 543 |
| polyamide 6 | 3511 | 3139 | 1627 | |
| high-density polyethylene | 4248 | 2130 | 2530 | 543 |
| polyester | 346 | 1815 | 543 | 9031 |

| | | | | |
|---|------|------|------|------|
| polyetherimide | 2363 | 742 | | 181 |
| vinyl ester | 145 | 1206 | | 181 |
| ethylene-vinyl acetate | 669 | 151 | | 181 |
| polyetheramine | 9452 | 9914 | 3251 | 5419 |
| ethylene chlorotrifluoroethylene (fluorocarbon) | 1974 | 2412 | 1626 | 542 |

Table 4 - Common polymers in the first and second pools

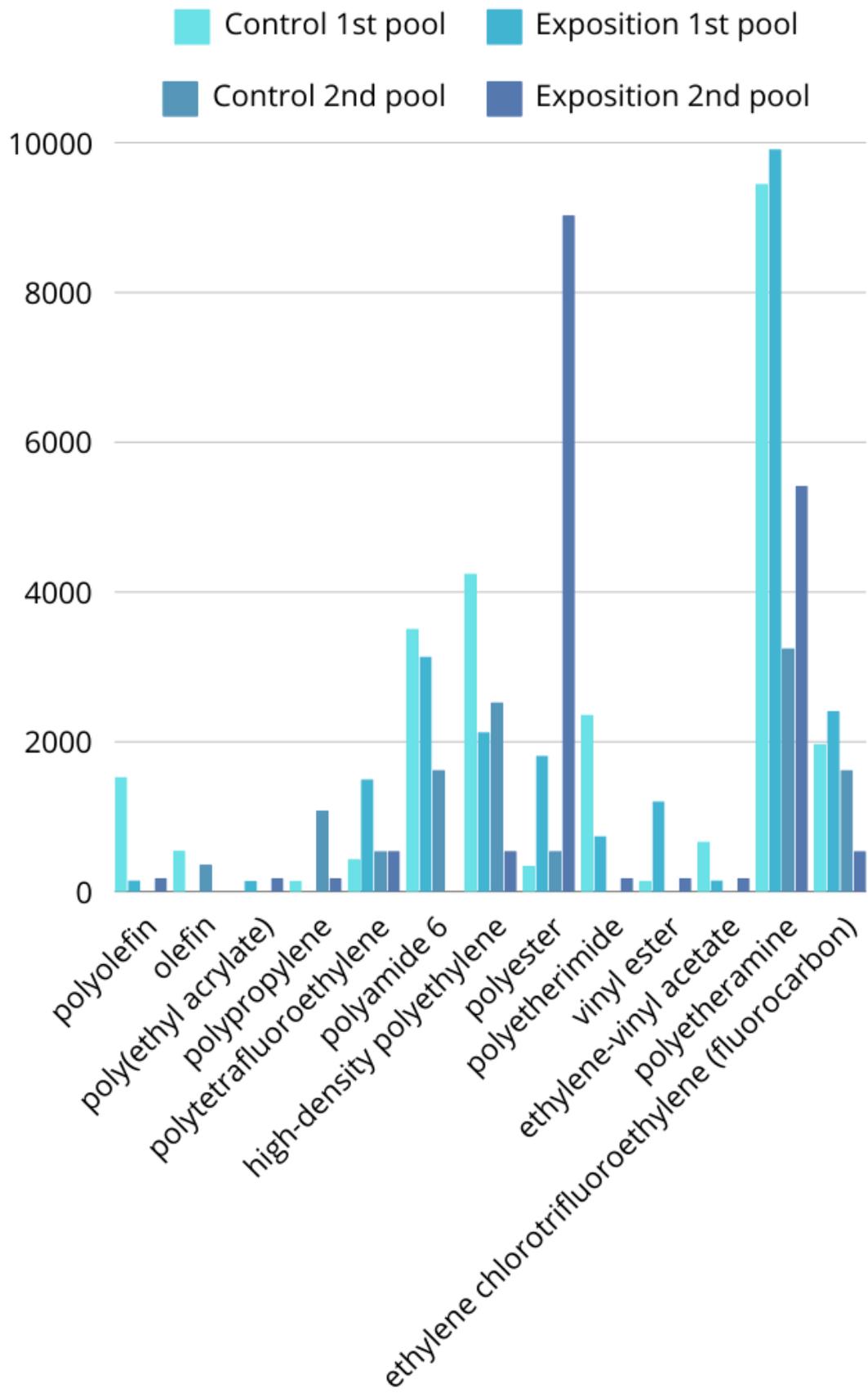


Figure 11 - Common polymers in the first and second pools

3.4 Comparison of water and mussels from the first mesocosm

There were six types of polymers in mussels from the first pool (table 1): olefin, polyamide 6, polypropylene, polyphthalamide, polyarylamide and modacrylic. Three of them were common microplastics in water (in experimental samples) and mussels from the first pool: polyamide 6, polypropylene and modacrylic, so they were ingested by mussels from the pool.

Olefin was found only in control samples, it was not found in any experimental sample. Polyphthalamide and polyarylamide were not isolated from the first pool, so maybe these microplastics were constantly present in mussels or were ingested before experiments or they entered the mesocosm during the days, because the seawater was daily added to the mesocosm so as that the volume in the tank was always the same.

Study of Woods et al., 2018 finds 71% of microplastic particles are rejected by mussels as pseudofeces. In this research pseudofeces were not analyzed. However, the size of microplastics in pseudofeces is above that which allows their passage from the gills to the digestive system (Corami et al., 2020b).

Since polyethylene particles were found in the control and mesocosm, it was difficult to understand whether mussels ingested these particles. Besides, it is possible that mussels mainly excreted aged microplastic particles with pseudofeces since their sizes may not be suitable for ingestion.

Polyamide 6 predominated in experimental samples and also it predominated in mussels (figure 12). Mussels ingested 395 from 3284 microplastic particles of polyamide 6, polypropylene and modacrylic. Also, they ingested 395 from 25567 all microplastic particles from experimental samples from the second pool.

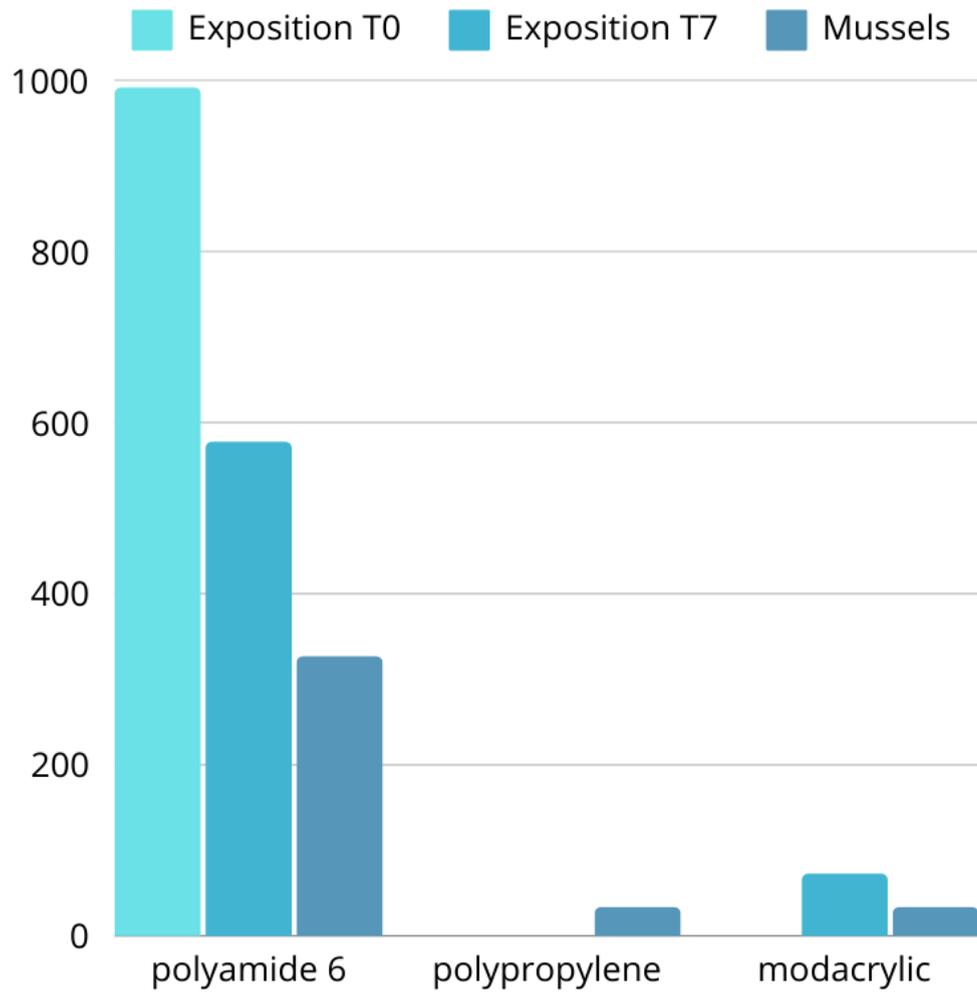


Figure 12 - Common microplastics in water and mussels in the first pool

3.5 Comparison of water and mussels from the second mesocosm

There were three types of microplastic in mussels from the first pool (table 1), all of them were isolated both from water and mussels from the second pool: poly(ethyl acrylate), polypropylene and polyethylene (table 6), so mussels ingested these microplastics from water. Abundance of these microplastics was several times higher in water than in mussels.

Polypropylene predominated in mussels (figure 13).

Mussels ingested 121 from 905 microplastic particles of poly(ethyl acrylate), polypropylene and polyethylene. Also, they ingested 121 from 17707 all microplastic particles from experimental samples from the second pool.

Mussels consumed 11% of microplastic types from the first pool and 18% microplastics from the second pool.

Mussels ingested polypropylene from the second and the first pool. In Digka et al., 2024 research polypropylene was found only in Marine Protected Areas while in the Mussel and Fish farm it was not found. In that research polypropylene, polyamide and polyethylene terephthalate were found in both places, so polymer composition was different.

In this study mussels ingested poly(ethyl acrylate) and polyethylene only from the second pool, despite the fact that there were these microplastics also in the first pool; but mussels ingested only polyamide 6, polypropylene and modacrylic from the first pool. So perhaps mussels always ingest polypropylene and ingest poly(ethyl acrylate) and polyethylene according to the polymer's size.

Polyamide 6 predominated in experimental samples and also it predominated in mussels in the first pool.

Mussels ingested 395 from 3284 SMPs/L of polyamide 6, polypropylene and modacrylic. They ingested 121 from 905 SMPs/L of poly(ethyl acrylate), polypropylene and polyethylene. Mussels consumed 395 from 25567 SMPs/L of all microplastic particles from experimental samples from the second pool, and 121 from 17707 SMPs/L of all microplastic particles from experimental samples from the second pool.

Although the ingestion of microplastics by mussels is not constant and can change due to temperature and oxygen level (Kankılıç et al., 2023), particles' size is definitely significant for the ingestion of SMPs by biota.

Bivalve mollusks are influenced directly and indirectly by microplastic (Khanjani et al., 2023). So, it is necessary to develop mitigation strategies and decision-making solutions, study ingestion of microplastics by different living organisms and monitoring analysis to reduce negative effects of microplastic pollution.

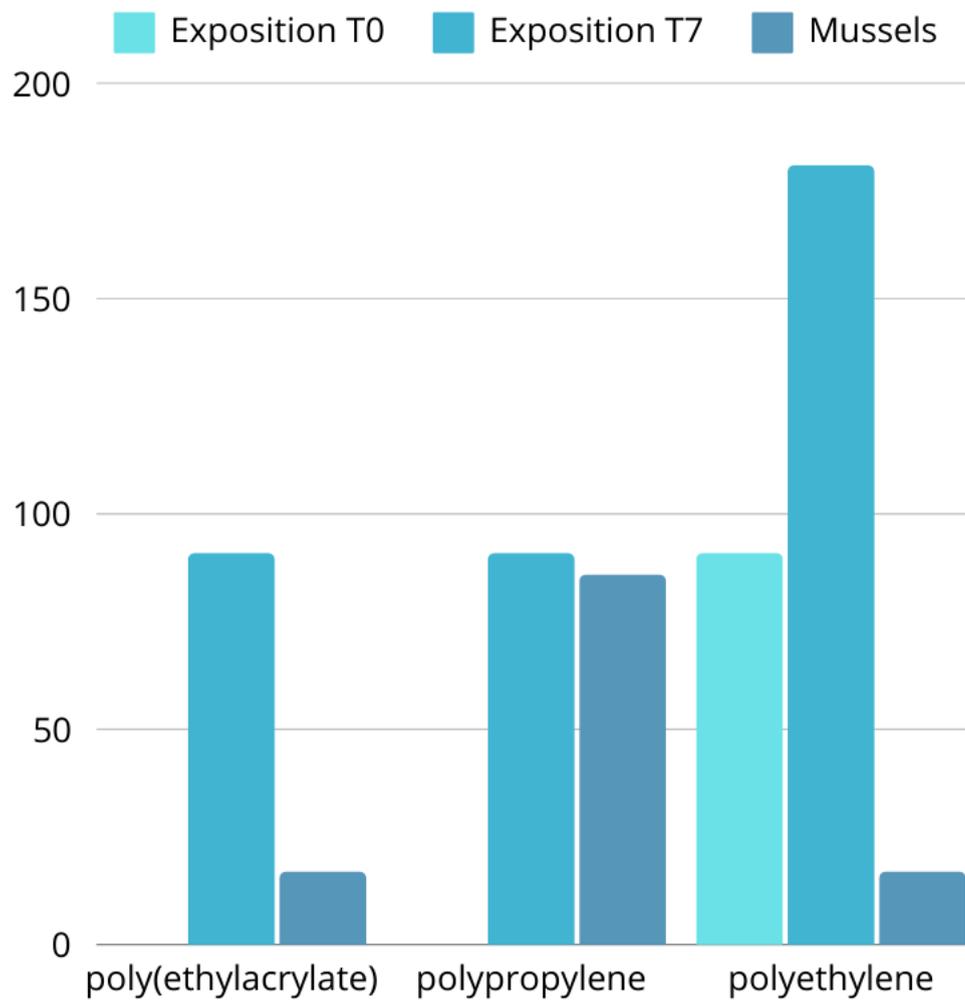


Figure 13 - Common polymers in water and mussels in the second pool

4 Conclusions

The objective of this research was to check whether aged microplastics may be ingested by mussels. It was observed that mussels ingested microplastics, but not specifically the aged microplastics added in the mesocosms.

Furthermore, the control 1 showed a significantly higher abundance of small microplastics than the exposure mesocosm 1, while the control 2 and the mesocosm 2 showed similar abundances at the start and at the end of the experiment. Specifically, polyester was found in the controls and in the two mesocosms. According to the results, it cannot be established that aged microplastics were ingested more than the other microplastics, since there are environmental microplastics, including polyester, in the seawater of the controls.

Size is the principal driver for the ingestion; hence, aged microplastics may have not been of the suitable size for ingestion and they were excreted with pseudofeces. Besides, the resuspension of polyester particles may have not been efficient and those aged particles may have been at the bottom of the tank and they were not completely recovered when collecting water at the start and at the end of the exposure experiment.

Future studies are necessary to set up some crucial variables such as the size of the aged particles, the resuspension of these particles inside the tank for the whole experiment, the quality assurance/quality control protocols to check and minimize the plastic contamination constantly throughout the experiment, the necessity to improve the quality of the seawater filtration and to cover the tanks better throughout the exposure experiments. Besides, the duration of the exposure should be increased to observe and effective ingestion by the mussels.

5 References

1. Álvarez-Ruiz, R., Picó, Y., Campo, J. (2021). Bioaccumulation of emerging contaminants in mussel (*Mytilus galloprovincialis*): Influence of microplastics. *Science of The Total Environment*. 796: 149006.
2. Anbumani, S., Kakkar, P. (2018). Ecotoxicological effects of microplastics on biota: a review. *Environmental Science and Pollution Research International*. 25(15):14373-14396.
3. Andrady, A. L. (2015). Persistence of plastic litter in the oceans. *Marine Anthropogenic Litter*. 57–72.
4. Andrady, A.L. (2017). The plastic in microplastics: A review. *Marine Pollution Bulletin*. 119(1): 12-22.
5. Aramendia, J., García-Velasco, N., Amigo, J.M., Izagirre, U., Seifert, A., Soto, M., Castro, K. (2024). Evidence of internalized microplastics in mussel tissues detected by volumetric Raman imaging. *Science of The Total Environment*. 914: 169960.
6. Baldwin, A.K., Spanjer, A.R., Rosen, M.R., Thom, T. (2020). Microplastics in Lake Mead National Recreation Area, USA: Occurrence and biological uptake. *PloS One*. 15(5):e0228896.
7. Bao, R., Cheng, Z., Hou, Y., Xie, C., Pu, J., Peng, L., Gao, L., Chen, W., Su, Y. (2022). Secondary microplastics formation and colonized microorganisms on the surface of conventional and degradable plastic granules during long-term UV aging in various environmental media. *Journal of Hazardous Materials*. 439: 129686.
8. Barboza, L.G.A., Vieira, L.R., Guilhermino, L. (2018). Single and combined effects of microplastics and mercury on juveniles of the European seabass (*Dicentrarchus labrax*): Changes in behavioural responses and reduction of swimming velocity and resistance time. *Environmental Pollution*. 236:1014-1019.
9. Barnes, D.K.A., Galgani, F., Thompson, R.C., Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environments. *Philos Trans R Soc Lond B Biol Sci*. 364(1526): 1985–1998.
10. Boucher, J., Friot, D. (2017). Primary Microplastics in the Oceans: A Global Evaluation of Sources. *The International Union for Conservation of Nature (IUCN)*. 43.
11. Bråte, I.L.N., Hurley, R., Iversen, K., Beyer, J., Thomas, K.V., Steindal, C.C., Green, N.W., Olsen, M., Lusher, A. (2018). *Mytilus* spp. as sentinels for monitoring microplastic pollution in Norwegian coastal waters: A qualitative and quantitative study. *Environmental Pollution*. 243(A): 383-393.

12. Browne, M.A., Dissanayake, A., Galloway, T.S., Lowe, D.M., Thompson, R.C. (2008). Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L). *Environmental science & technology*. 42(13):5026-31.
13. Carr, S.A., Liu, J., Tesoro, A.G. (2016). Transport and fate of microplastic particles in wastewater treatment plants. *Water Research*. 91: 174-182.
14. Cincinelli, A., Scopetani, C., Chelazzi, D., Lombardini, E., Martellini, T., Katsoyiannis, A., Fossi, M.C., Corsolini, S. (2017). Microplastic in the surface waters of the Ross Sea (Antarctica): Occurrence, distribution and characterization by FTIR. *Chemosphere*. 175: 391-400.
15. Cole, M., Lindeque, P., Halsband, C., Galloway, T.S. (2011). Microplastics as contaminants in the marine environment: A review. *Marine Pollution Bulletin*. 62(12): 2588-2597.
16. Corami, F., Rosso, B., Bravo, B., Gambaro, A., Barbante, C. (2020a). A novel method for purification, quantitative analysis and characterization of microplastic fibers using Micro-FTIR. *Chemosphere*. 238:124564.
17. Corami, F., Rosso, B., Iannilli, V., Ciadamidaro, S., Bravo, B., Barbante, C. (2022a). Occurrence and Characterization of Small Microplastics (<100 μm), Additives, and Plasticizers in Larvae of Simuliidae. *Toxics*. 10(7):383.
18. Corami, F., Rosso, B., Morabito, E., Rensi, V., Gambaro, A., Barbante, C. (2021). Small microplastics (<100 μm), plasticizers and additives in seawater and sediments: Oleo-extraction, purification, quantification, and polymer characterization using Micro-FTIR. *Science of the Total Environment*. 797:148937.
19. Corami, F., Rosso, B., Roman, M., Picone, M., Gambaro, A., Barbante, C. (2020b). Evidence of small microplastics (<100 μm) ingestion by Pacific oysters (*Crassostrea gigas*): A novel method of extraction, purification, and analysis using Micro-FTIR. *Marine pollution bulletin*. 160:111606.
20. Corami, F., Rosso, B., Sfriso, A.A., Gambaro, A., Mistri, M., Munari, C., Barbante, C. (2022b). Additives, plasticizers, small microplastics (<100 μm), and other microlitter components in the gastrointestinal tract of commercial teleost fish: Method of extraction, purification, quantification, and characterization using Micro-FTIR. *Marine Pollution Bulletin*. 177:113477.
21. Crawford, C.B., Quinn, B. (2017). The interactions of microplastics and chemical pollutants. *Microplastic Pollutants*. 131-157.
22. Devereux, R., Westhead, E.K., Jayaratne, R., Newport, D. (2022). Microplastic abundance in the Thames River during the New Year period. *Marine pollution bulletin*. 177:113534.

23. Digka, N., Patsiou, D., Hatzonikolakis, Y., Raitzos, D.E., Skia, G., Koutsoubas, D., Dimitriadis, C., Tsangaris, C. (2024). Microplastic ingestion in mussels from the East Mediterranean Sea: Exploring its impacts in nature and controlled conditions. *Science of The Total Environment*. 946:174268.
24. ECHA (European Chemicals Agency)
25. Annex XV Restriction Report Proposal for a Restriction
26. Report version number 1 (March 20th 2019). Helsinki
27. (2019)
28. ElMasry, G., Sun, D.-W. (2010). CHAPTER 6 - Meat Quality Assessment Using a Hyperspectral Imaging System. *Hyperspectral Imaging for Food Quality Analysis and Control*. 175-240.
29. Eriksen, M., Lebreton, L.C.M., Carson, H.S., Thiel, M., Moore, C.J., Borerro, J.C., Galgani, F., Ryan, P.G., Reisser, J. (2014). Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. *PLoS One*. 9(12):e111913.
30. Frias, J.P.G.L., Nash, R. (2019). Microplastics: Finding a consensus on the definition. *Marine Pollution Bulletin*. 138:145-147.
31. Gamarro, E.G., Rojas, D.L.S., Martínez, R.M.G., González, G.P., Hernando, P.F. (2024). Occurrence of common plastic additives and contaminants in raw, steamed and canned mussel samples from different harvesting areas using MSPD-HPLC methodology. *Food Research International*. 181: 114109.
32. Geyer, R., Jambeck, J.R., Law, K.L. (2017). Production, use, and fate of all plastics ever made. *Science Advances*. 3(7):e1700782.
33. Hale, R.C., Seeley, M. E., La Guardia, M. J., Mai, L., Zeng., E.Y. (2020). A Global Perspective on Microplastics. *Journal of Geophysical Research: Oceans*. 125(1), doi: 10.1029/2018JC014719.
34. Hanvey, J.S., Lewis, P.J., Lavers, J.L., Crosbie, N.D., Pozo, K., Clarke, B.O. (2017). A review of analytical techniques for quantifying microplastics in sediments. *Analytical Methods*. 9: 1369-1383.
35. He, B., Wijesiri, B., Ayoko, G.A., Egodawatta, P., Rintoul, L., Goonetilleke, A. (2020). Influential factors on microplastics occurrence in river sediments. *Science of The Total Environment*. 738: 139901.
36. Hidalgo-Ruz, V., Gutow, L., Thompson, R. C., Thiel, M. (2012). Microplastics in the marine environment: a review of the methods used for identification and quantification. *Environmental Science & Technology*. 20; 46(6): 3060-75.

37. Horton, A.A., Weerasinghe, K.D.I., Mayor, D.J., Lampitt, R. (2024). Microplastics in commercial marine fish species in the UK – A case study in the River Thames and the River Stour (East Anglia) estuaries. *Science of The Total Environment*. 915: 170170.
38. Hurley, R., Woodward, J., Rothwell, J.J. (2018). Microplastic contamination of river beds significantly reduced by catchment-wide flooding. *Nature Geoscience*. 11: 251–257.
39. Iannilli, V., Corami, F., Grasso, P., Lecce, F., Buttinelli, M., Setini, A. (2020). Plastic abundance and seasonal variation on the shorelines of three volcanic lakes in Central Italy: can amphipods help detect contamination? *Environmental Science and Pollution Research*. 27:14711–14722.
40. Iannilli, V., Pasquali, V., Setini, A., Corami, F. (2019). First evidence of microplastics ingestion in benthic amphipods from Svalbard. *Environmental Research*. 179(Pt A): 108811.
41. Imhof, H.K., Sigl, R., Brauer, E., Feyl, S., Gieseemann, P., Klink, S., Leupolz, K., Löder, M.G.J., Löschel, L.A., Missun, J., Muszynski, S., Ramsperger, A.F.R.M., Schrank, I., Speck, S., Steibl, S., Trotter, B., Winter, I., Laforsch, C. (2017). Spatial and temporal variation of macro-, meso- and microplastic abundance on a remote coral island of the Maldives, Indian Ocean. *Marine pollution bulletin*. 116(1-2):340-347.
42. Jong, M.-C., Li, J., Noor, H.M., He, Y., Gin, K.Y.-H. (2022). Impacts of size-fractionation on toxicity of marine microplastics: Enhanced integrated biomarker assessment in the tropical mussels, *Perna viridis*. *Science of The Total Environment*. 835: 155459.
43. Junaid, M., Siddiqui, J.A., Sadaf, M., Liu, S., Wang, J. (2022). Enrichment and dissemination of bacterial pathogens by microplastics in the aquatic environment. *Science of The Total Environment*. 830, 154720.
44. Kankılıç, G.B., Koraltan, İ., Erkmén, B., Çağan, A.S., Çırak, T., Özen, M., Seyfe, M., Altındağ, A., Tavşanoğlu, Ü.N. (2023). Size-selective microplastic uptake by freshwater organisms: Fish, mussel, and zooplankton. *Environmental Pollution*. 336:122445.
45. Katlam, G., Prasad, S., Pande, A., Ramchiary, N. (2022). Plastic ingestion in Asian elephants in the forested landscapes of Uttarakhand, India. *Journal for Nature Conservation*. 68: 126196.
46. Kelleher, L., Schneidewind, U., Krause, S., Haverson, L., Allen, S., Allen, D., Kukkola, A., Murray-Hudson, M., Maselli, V., Franchi, F. (2023). Microplastic accumulation in endorheic river basins - The example of the Okavango Panhandle (Botswana). *The Science of the total environment*. 874: 162452.
47. Khalid, A., Zalouk-Vergnoux, A., Benali, S., Mincheva, R., Raquez, J.-M., Bertrand, S., Poirier, L. (2021). Are bio-based and biodegradable microplastics impacting for blue mussel (*Mytilus edulis*)? *Marine Pollution Bulletin*. 167: 112295.

48. Khanjani, M.H., Sharifinia, M., Mohammadi, A.R. (2023). The impact of microplastics on bivalve mollusks: A bibliometric and scientific review. *Marine Pollution Bulletin*. 194(A): 115271
49. Khoironi, A., Anggoro, S., Sudarno, U. (2018). The existence of microplastic in Asian green mussels. *IOP Conference Series: Earth and Environmental Science*. 131(1): 012050.
50. Kirstein, I.V., Kirmizi, S., Wichels, A., Garin-Fernandez, A., Erler, R., Löder, M., Gerdts, G. (2016). Dangerous hitchhikers? Evidence for potentially pathogenic *Vibrio* spp. on microplastic particles. *Marine Environmental Research*. 120: 1-8.
51. Kolandhasamy, P., Su, L., Li, J., Qu, X., Jabeen, K., Shi, H. (2018). Adherence of microplastics to soft tissue of mussels: A novel way to uptake microplastics beyond ingestion. *The Science of the total environment*. 610–611: 635-640.
52. Laursen, S.N., Fruergaard, M., Dodhia, M.S., Posth, N.R., Rasmussen, M.B., Larsen, M.N., Shilla, D., Shilla, D., Kilawe, J.J., Kizenga, H.J., Andersen, T.J. (2023). Settling of buoyant microplastic in estuaries: The importance of flocculation. *The Science of the total environment*. 886:163976.
53. Li, J., Liu, H., Chen, J.P. Microplastics in freshwater systems: A review on occurrence, environmental effects, and methods for microplastics detection. (2018). *Water Research*. 137: 362-374.
54. Li, J., Lusher, A.L., Rotchell, J.M., Deudero, S., Turra, A., Bråte, I.L.N., Sun, C., Hossain, M.S., Li, Q., Kolandhasamy, P., Shi, H. (2019). Using mussel as a global bioindicator of coastal microplastic pollution. *Environmental Pollution*. 244: 522-533.
55. Li, H., Yang, Z., Jiang, F., Li, L., Li, Y., Zhang, M., Qi, Z., Ma, R., Zhang, Y., Fang, J., Chen, X., Geng, Y., Cao, Z., Pan, G., Yan, L., Sun, W. (2023). Detection of microplastics in domestic and fetal pigs' lung tissue in natural environment: A preliminary study. *Environmental Research*. 216(2): 114623.
56. Malla-Pradhan, R., Suwunwong, T., Phoungthong, K., Joshi, T.P., Pradhan, B.L. (2022). Microplastic pollution in urban Lake Phewa, Nepal: the first report on abundance and composition in surface water of lake in different seasons. *Environmental science and pollution research international*. 29(26):39928-39936.
57. Mariano, S., Tacconi, S., Fidaleo, M., Rossi, M., Dini, L. (2021). Micro and Nanoplastics Identification: Classic Methods and Innovative Detection Techniques. *Frontiers in Toxicology*. 3: 636640.
58. McCormick, A., Hoellein, T.J., Mason, S.A., Schlupe, J., Kelly, J.J. (2014). Microplastic is an abundant and distinct microbial habitat in an urban river. *Environmental science & technology*. 48(20):11863-71.

59. Mecozzi, M., Pietroletti, M., Monakhova, Y.B. (2016). FTIR spectroscopy supported by statistical techniques for the structural characterization of plastic debris in the marine environment: Application to monitoring studies. *Marine Pollution Bulletin*. 106(1-2): 155-161.
60. Meijer, L.J.J., van Emmerik, T., van der Ent, R., Schmidt, C., Lebreton, L. (2021). More than 1000 rivers account for 80% of global riverine plastic emissions into the ocean. *Science Advances*. 7(18): eaaz5803.
61. Nematollahi, M.J., Moore, F., Keshavarzi, B., Vogt, R.D., Saravi, H.N., Busquets, R. (2020). Microplastic particles in sediments and waters, south of Caspian Sea: Frequency, distribution, characteristics, and chemical composition. *Ecotoxicology and environmental safety*. 206:111137.
62. Oktavilia, S., Hapsari, M., Firmansyah, Setyadharma, A., Wahyuningsum, I.F.S. (2020). Plastic Industry and World *Environmental Problems*. 202, 05020.
63. Our World in Data, Plastic Pollution Data. <https://ourworldindata.org/plastic-pollution>
64. Patterson, J., Jeyasanta, K.I., Laju, R.L., Edward, J.K.P. (2021). Microplastic contamination in Indian edible mussels (*Perna perna* and *Perna viridis*) and their environs. *Marine Pollution Bulletin*. 171: 112678.
65. Peñalver, R., Arroyo-Manzanares, N., López-García, I., Hernández-Córdoba, M. (2020). An overview of microplastics characterization by thermal analysis. *Chemosphere*. 242: 125170.
66. Phothakwanpracha, J., Lirdwitayaprasit, T., Pairohakul, S. (2021). Effects of sizes and concentrations of different types of microplastics on bioaccumulation and lethality rate in the green mussel, *Perna viridis*. *Marine Pollution Bulletin*. 173: 112954.
67. Prata, J.C., Costa, J.P., Lopes, I. Duarte, A.C., Rocha-Santos, T. (2020). Environmental exposure to microplastics: An overview on possible human health effects. *Science of The Total Environment*. 702, 134455.
68. Primpke, S., Wirth, M., Lorenz, C., Gerdt, G. (2018). Reference database design for the automated analysis of microplastic samples based on Fourier transform infrared (FTIR) spectroscopy. *Analytical and Bioanalytical Chemistry*. 410(21): 5131-5141.
69. Qu, X., Su, L., Li, H., Liang, M., Shi, H. (2018). Assessing the relationship between the abundance and properties of microplastics in water and in mussels. *The Science of the total environment*. 621:679-686.
70. Rheinberger, C., Perrti Elo, P., Henrichson, S., Kapanen, A., Lefevre-Brevart, S., Majoros, L., Stoyanova, E., Simpson, P. (2021). Regulating primary microplastics in the European Union.

71. Rimondi, V., Monnanni, A., De Beni, E., Bicchocchi, G., Chelazzi, D., Cincinelli, A., Fratini, S., Martellini, T., Morelli, G., Venturi, S., Lattanzi, P., Costagliola, P. (2022). Occurrence and Quantification of Natural and Microplastic Items in Urban Streams: The Case of Mugnone Creek (Florence, Italy). *Toxics*. 10(4):159.
72. Rohrbach, S., Gkoutselis, G., Hink, L., Weig, A.R., Obst, M., Diekmann, A., Ho, A., Rambold, G., Horn, M.A. (2023). Microplastic polymer properties as deterministic factors driving terrestrial plastisphere microbiome assembly and succession in the field. *Environmental microbiology*. 25(12):2681-2697
73. Rossatto, A., Arlindo, M.Z.F., de Moraes, M.S. de Souza, T.D., Ogrodowski, C.S. (2023). Microplastics in aquatic systems: A review of occurrence, monitoring and potential environmental risks. *Environmental Advances*. 13, 100396.
74. Srisiri, S., Haetrakul, T., Dunbar, S.G., Chansue, N. (2024). Microplastic contamination in edible marine fishes from the upper Gulf of Thailand. *Marine Pollution Bulletin*. 198: 115785.
75. Tokunaga, Y., Okochi, H., Tani, Y., Niida, Y., Tachibana, T., Saigawa, K., Katayama, K., Moriguchi, S., Kato, T., Hayama, S. (2023). Airborne microplastics detected in the lungs of wild birds in Japan. *Chemosphere*. 321: 138032.
76. Villarrubia-Gómez, P., Cornell, S.E., Fabres, J. (2018). Marine plastic pollution as a planetary boundary threat – The drifting piece in the sustainability puzzle. *Marine Policy*. 96: 213-220.
77. von der Esch, E., Lanzinger, M., Kohles, A.J., Schwaferts, C., Weisser, J., Hofmann, T., Glas, K., Elsner, M., Ivleva, N.P. (2020). Simple Generation of Suspensible Secondary Microplastic Reference Particles via Ultrasound Treatment. *Frontiers in chemistry*. 8:169.
78. Wang, W., Zhang, J., Qiu, Z., Cui, Z., Li, N., Li, X., Wang, Y., Zhang, H., Zhao, C. (2022). Effects of polyethylene microplastics on cell membranes: A combined study of experiments and molecular dynamics simulations. *Journal of Hazardous Materials*. 429: 128323.
79. Wayman, C., González-Pleiter, M., Fernández-Piñas, F., Sorribes, E.L., Fernández-Valeriano, R., López-Márquez, I., González-González, F., Rosal, R. (2024). Accumulation of microplastics in predatory birds near a densely populated urban area. *Science of The Total Environment*. 917: 170604.
80. Woods, M.N., Stack, M.E., Fields, D.M., Shaw, S.D., Matrai, P.A. (2018). Microplastic fiber uptake, ingestion, and egestion rates in the blue mussel (*Mytilus edulis*). *Marine Pollution Bulletin*. 137: 638-645.
81. Xia, B., Sui, Q., Du, Y., Wang, L., Jing, J., Zhu, L., Zhao, X., Sun, X., Booth, A.M., Chen, B., Qu, K., Xing, B. (2022). Secondary PVC microplastics are more toxic than primary PVC

- microplastics to *Oryzias melastigma* embryos. *Journal of hazardous materials*. 424(Pt B):127421.
82. Xu, J.-L., Thomas, K. V., Luo, Z., Gowen, A. A. (2019). FTIR and Raman imaging for microplastics analysis: State of the art, challenges and prospects. *TrAC Trends in Analytical Chemistry*. 119, 115629.
83. Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A. (2013). Life in the "plastisphere": microbial communities on plastic marine debris. *Environmental Science & Technology*. 47(13):7137-46.