UNIVERZA NA PRIMORSKEM
FAKULTETA ZA MATEMATIKO, NARAVOSLOVJE IN INFORMACIJSKE TEHNOLOGIJE

MASTER'S THESIS
(MAGISTRSKO DELO)

USE OF THE MEDITERRANEAN MUSSEL (*Mytilus galloprovincialis*) FILTRATION FUNCTION AS A SUSTAINABLE TOOL FOR WATER COLUMN MICROPLASTIC MONITORING

UPORABA FILTRACIJSKE FUNKCIJE UŽITNE KLAPAVICE (*Mytilus galloprovincialis*) KOT TRAJNOSTNO ORODJE ZA SPREMLJANJE MIKROPLASTIKE V VODNEM STOLPCU

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Use of the Mediterranean mussel (*Mytilus galloprovincialis*) filtration function as a sustainable tool for water column microplastic monitoring

(Uporaba filtracijske funkcije užitne klapavice *Mytilus galloprovincialis* kot trajnostno orodje za spremljanje mikroplastike v vodnem stolpcu)

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Izvleček:

V sodobnem svetu plastični odpadki predstavljajo eno izmed največjih okoljskih težav in izzivov. Literatura in naše raziskave so pokazale, da je najpogostejši plastični odpadek na plažah po svetu in v Sloveniji rabljen cigaretni ogorek, ki predstavlja mehansko in kemično nevarnost morskemu ekosistemu. Zaradi lahke dostopnosti, splošnega pojavljanja v vzhodnem Jadranskem morju in dobrega poznavanja biologije in splošnega gojenja je bila za modelni organizem izbrana užitna klapavica (*Mytilus galloprovincialis*). V sklopu magistrske naloge smo v laboratorijskih pogojih raziskovali, ali lahko filtratorsko funkcijo užitne klapavice uporabimo kot trajnostno orodje za spremljanje prisotnosti mikroplastike v vodnem stolpcu. Rezultati so pokazali, klapavice 90% vseh mikrovlaken izločijo v obliki fekalnega in psevdo-fekalnega peleta kar se je izkazalo za efektivno metodo za zbiranje in situ vzorcev na gojiščih školjk. Podatki raziskave so pomembni za prihodnje raziskovanje uporabe školjk kot bioindikatorjev in potencialnega orodja za odstranjevanje mikroplastike iz vodnega stolpca na večji prostorski ravni. Prav tako podatki pomembno prispevajo k razvoju uspešnejše metode za zaznavo in monitoring mikroplastike v vodnih okoljih.

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Abstract:

Plastic waste is one of the most significant environmental problems and challenges in the modern world. Literature and our research have shown that the most common plastic waste on beaches worldwide and in Slovenia is used cigarette butt, which poses a mechanical and chemical threat to the marine ecosystem. Due to its easy accessibility, widespread occurrence in the eastern Adriatic Sea, and extensive knowledge of its biology and general cultivation, we selected the edible mussel (*Mytilus galloprovincialis*) as the model organism. As part of this master's thesis, we investigated in laboratory conditions whether the filtering function of the edible mussel could be used as a sustainable tool for monitoring the presence of microplastics in the water column. The results showed that mussels excrete 90% of all microfibers as faecal and pseudo-faecal pellets, which proved an effective method for collecting in situ samples at mussel farms. The research data are essential for future studies on the use of mussels as bioindicators and as a potential tool for removing microplastics from the water column on a larger spatial scale. Additionally, the data significantly contribute to developing more effective methods for detecting and monitoring microplastics in aquatic environments.

ACKNOWLEDGEMENTS

The work reported in this thesis has been carried out in the Laboratory of Marine Nanotechnology and Biotechnology at the Ruđer Bošković Institute, Center for Marine Research, Rovinj, Croatia, under the leadership of prof. Daniel Mark Lyons, PhD. This research was conducted within the framework of the significant project MARELESS (Interreg Italy – Croatia, MARine Litter cross-border awarenESS and innovation), which profoundly impacted our understanding of marine litter.

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I want to express my heartfelt gratitude to my mentor, Bojan Hamer, and co-mentor, Dijana Pavičić-Hamer, who made several months of internship in 2021 at the Centre for Marine Research possible despite the challenges of the pandemic. I am truly grateful for all the professional and moral support during the research and the preparation of the written work. They are mentors in the truest sense of the word, and I am thankful for all the priceless experiences they gave me. Furthermore, I would like to thank all my laboratory co-workers for welcoming me as an equal team member and for all the assistance, guidance, and unforgettable moments we shared in Rovinj.

I would also like to thank my co-mentor, prof. Elena Bužan, for her kind guidance and all the professional support.

A special thanks go to the Landscape Park Strunjan and Luka Preložnik for the beautiful close-up photos of the mussel's farm and their display with cigarette butts.

Finally, I would like to express my deepest gratitude to my family, my husband, and to all my friends. Your unwavering support, kind words, advice, and occasional nudges in the right direction have been my anchor throughout this journey. I am truly grateful for your patience and understanding, and I could not have completed this journey without you.

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LIST OF ABBREVIATIONS

CB – cigarette butts
CBU – used (smoked) cigarette butts
CI – condition index
dH_20 – distilled water
MP(s) – microplastic(s)
MMS – Mussel Microplastic biomonitoring and purification System
Mt – metric tones
PAH – polycyclic aromatic hydrocarbons
PE-HD – polyethylene high density
PE-LD – polyethylene low density
PET – polyethylene terephthalate
PP – polypropylene
PVC – polyvinyl chloride
PW – plastic waste

1. INTRODUCTION

Plastic pollution has attracted significant attention from scientists and the general public in the past decades. Plastic items are a significant component of waste, accumulating extensively from the deepest parts of the ocean to the most remote areas of the Earth. Over recent decades, the widespread use of plastics and inadequate management of plastic waste (PW) has led to a troubling buildup of plastic debris in oceans, seas, and coastal areas worldwide. PW presents mechanical and chemical hazards to all living organisms, making regular monitoring essential for assessing the extent of PW and evaluating the effectiveness of strategies to limit or reduce PW in the environment (Ryan et al. 2009).

Plastics, as waste, dominate in all environments, and the marine environment is no exception. Plastic particles can be found at the sea surface, washed up on the shorelines, on the sea floor, and in the water column itself (Galgani et al. 2015; Li et al. 2016). Galgani et al. (2015) say plastic sometimes represents 100% of floating litter. Plastic litter in the ocean has emerged as one of our time's most pressing environmental challenges. As the world grapples with escalating levels of plastic pollution, the impact on marine ecosystems and biodiversity has become alarmingly evident.

The word "plastics" indicates a group of synthetic or organic synthetic polymers with common properties: low price, light in weight, resilience, and non-corrosiveness. Plastics have become integral to our daily lives, revolutionizing industries and transforming how we live, work, and consume. As a versatile and durable material, plastics have found their way into countless applications, from packaging and construction to electronics and healthcare (Li et al. 2016). Unfortunately, those highly valued properties in the industry are the most problematic for the environment because all types of plastics become very brittle over time and break into smaller particles. Additionally, plastics break down under the influence of UV light and seawater. However, we still need to determine how long precisely plastic takes to decompose as the decomposition time entirely depends on the type of plastic itself (Li et al. 2016).

Although plastic debris is a global issue, despite many voices raised on the issue, we need more consistency in defining and categorizing PW, which can lead to ambiguous communication and the generation of incomparable data (Hartmann et al. 2019). Hartmann et al. (2019) proposed a framework which differentiates between defining criteria that address basic properties and additional criteria. They suggest that chemical composition, solid state and solubility, size, shape, structure, colour and origin should be considered.

According to Napper and Thompson (2023), global use of plastics increased from 5 million metric tons (Mt) in the 1950s to 367 million Mt in 2020 and the amount of PW. PlasticsEurope (2020) stated that the most commonly used plastic polymers are PVC, PP,

PET, PE-HD and PE-LD. Common examples of land-based plastic pollution include plastic bags, bottles, packaging materials, and single-use plastics such as straws and cutlery, representing as much as 80% of all PW (Li et al. 2016). These items often enter waterways through inadequate disposal practices, stormwater runoff, or improper waste management systems. However, it is essential to recognize that plastic litter also results from marine-based activities. Fishing gear, such as lost or discarded nets and lines, accounts for a substantial portion of marine litter. These items, commonly referred to as "ghost gear," pose a severe threat to marine life, entangling and injuring numerous marine species, and they form around 20% of the total yield of marine PW (Li et al. 2016).

One of the most used criteria for plastic debris categorization is size because of its significant ecological relevance, as it will determine interaction with biota and its environmental fate as well as monitoring and potential remediation methods (Hartmann et al. 2019). When reviewing the various scientific literature, it is possible to notice that the size spans differ in their definitions. Most authors list categories with size prefixes such as nano-, micro-, meso- and macro-. By most of the authors, “macroplastics” is a term which indicates plastic particles more significant than 20 mm, “mesoplastics” are considered as plastics in a size range from 5 to 20 mm, particles smaller than 5 mm are considered “microplastics” (MPs) Some of the authors consider plastic particles smaller than 100 nm as “nanoparticles” (Valentić 2018; Hartmann et al. 2019).

1.1 Microplastics (MPs)

This thesis will focus on MPs and potential methods for biomonitoring and sustainable tools for removing them from the environment. Furthermore, its origin and environmental threats will be described in more detail. MPs consist of a diverse collection of particles that differ in size, shape, colour, chemical makeup, density, and other properties, as is their origin (Galgani et al. 2015).

Primary MPs are deliberately manufactured as microbeads and are commonly found in personal care products or used as raw materials for other manufacturing processes. These tiny pellets can enter the environment at various stages, including during production, transportation, or usage (Andrady 2017). On the other hand, secondary MPs consist of particles that result from the fragmentation of larger plastic items, such as synthetic fabrics

and are primarily formed through the breakdown of microplastics (Li et al. 2016; Andrady 2017). Distribution of MPs however exhibits significant heterogeneity. The transport dynamics and subsequent distribution and accumulation of MPs in different marine areas can be influenced by a multitude of factors. These factors include the physicochemical properties of MPs, such as their size, specific density, charge, and chemical composition, as well as hydrodynamic elements and environmental characteristics like water current velocity, turbidity, water mass density, temperature, and wind (Guzzetti et al. 2018).

Marine organisms can interact with microplastics through adhesion, absorption, ventilation or ingestion (Lusher et al. 2017). Numerous studies have provided evidence that once ingested, MPs can accumulate within organisms and be translocated between various body tissues. Additionally, MPs can be eliminated from organisms through excretion or egestion via pseudo-faeces (Guzzetti et al. 2018). Accumulated MPs can have a direct effect on the organisms that ingested them. Some of these include internal and external injuries, blockages of the digestive tract, physiological stress, changes in feeding behaviour, stunted growth and reduction in fertility, fecundity and survival rate (Guzzetti et al., 2018).

In addition to mechanical threats, MP particles can act as a vector for the introduction of toxic compounds in marine organisms, which can be added as additives in a production stage, or as environmental contaminants absorbed on the surface by the marine environment (Guzzetti et al. 2018). Those compounds can have a variety of harmful effects, such as alternation of metabolic and reproduction activity, oxidative stress, cellular activity, inflammation and cancer. Along the MP particles themselves, they can be transferred along the food chain through bioaccumulation and biomagnification (Guzzetti et al. 2018). At the same time, MP particles can act as a vessel for the transport and spread of various marine organisms in habitats where they could be recognized as invasive species (Guzzetti et al. 2018).

Another aspect of MP pollution is marine food safety, which is widely discussed by Walkinshaw et al. (2020). According to FAO (2024), 743 100 t of fishing organisms were caught between 2018 and 2020 in the Mediterranean Sea, which is 2.9% more than between 2014 and 2016. According to Hatoro et al. (2019), MPs have been discovered in sediments and waters of a wide variety of coastal areas around the globe, from densely populated areas to more remote areas, as well as in seafood. MPs have been found in all commercially important organisms, caught and farmed, which are fish, shellfish, crustaceans and even macroalgae; it is suggested that organisms which are positioned on a base of the food chain,

such as bivalves and crustaceans, are more likely to contain a higher amount of MPs in comparison with organisms on a higher trophic level (Walkinshaw et al. 2020) which is contributed to feeding strategy of shellfish which most of them are filter feeders (Hatoro et al. 2019). In addition to available data, it highlights the fact that laboratory research is difficult due to the high possibility of sample contamination with MP fibers, which are one of the most common types of MP debris worldwide (Walkinshaw et al. 2020; Bendell et al. 2020).

Measuring the cost of microplastic pollution to ecosystem services such as food provisioning through fisheries and aquaculture is very challenging. Monitoring itself is crucial to assess the efficiency of implemented measurements to reduce the abundance of PW, but effective monitoring is a challenge due to the large spatial and temporal heterogeneity of plastics, as well as the diversity of chemical structure and other factors (Ryan et al. 2009). To date, most of the research has focused on beach litter.

1.2 Cigarette butts – the most common beach litter

Beaches all over the world are saturated with anthropogenic litter, especially in populated areas as well as in more remote, isolated areas. Although beach litter is composed of various materials, PW dominates it. The most frequently collected PW during beach clean-ups is used cigarette butts (CB) (Araújo and Costa 2019). It is estimated that between one and two-thirds of the CB from smoked cigarettes are discarded in nearby environments by smokers, amounting to approximately 900 Mt each year (Dobaradarn et al. 2019).

CB contains 4 main components which are: burner or unburned tobacco, ashes, paper and a filter which is consisted of cellulose acetate (Araújo and Costa 2019). Cellulose itself is biodegradable material, but due to modification of the polymer by chemical processes called acetylation where plasticizers are added, cellulose acetate has limited potential for biodegradation and therefore it can be categorized as plastics (Araújo and Costa 2019).

In addition, there are more than 500 compounds present in cigarettes, among which at least 150 are considered highly toxic, carcinogenic and mutagenic (Araújo and Costa 2019). Acetone, arsenic, cadmium, carbon monoxide and lead (Adorati 2016). The toxic compounds may leach from different parts of CB, including filter and tobacco residue, which have the potential to contaminate water bodies (Dobradarn et al. 2019). CB posed mechanical and chemical threats to marine biota, as well as a source of microplastic fibers and was found in the stomach content of marine fauna (fish, birds, whales) (Araújo and Costa 2019).

As a part of the research for this thesis, we conducted a brief survey of the two popular Slovenian beaches, revealing that cigarette butts are the most common beach litter also on the Slovenian coast. The two beaches examined differ in that Svetilnik Beach is regularly cleaned by a municipal company, whereas Bele Skale Beach, being part of a landscape park Strunjan, does not receive regular cleaning. The examination of these beaches was conducted during the late afternoon during the summer season (2021). On both beaches, the most prevalent litter was used cigarette butts (Figure 1).

Figure 1: Used cigarette butts were found in the first 50 meters of the 400 meters sample section in total. Location: Bele Skale Beach (Landscape Park Strunjan),

Based on several studies provided data about the critical influence of smoked CB toxicity on the environment, MP fibers are the most common type of MPs in the environment and smoked CB possibly presents one of the sources of MP fibers in the environment (Moroz et al. 2021) and our own preliminary research, we decided that microplastic fibers derived from CB is the most suitable as the primary model for further research activities focused on sustainability and the effective removal of MPs from the environment.

1.3 *Mytilus* spp. as bioindicator

Mussels are members of several families of filter-feeding molluscs whose habitats range from freshwater to saltwater. However, the term “mussels” is most frequently used to refer to the edible bivalves of the marine family Mytilidae (Dailianis 2011). Most species of the Mytilidae family live on exposed shores in the intertidal zone and are of great human interest, as they are often intensively fished and cultured worldwide for human consumption (Dailianis 2011).

Genetic analysis discovered nine different species of the genus *Mytilus*, where the most common and cultured are the blue mussel (*Mytilus edulis*) and Mediterranean mussel (*Mytilus galloprovincialis*) (Dailianis 2011). In most marine mussels, the shell is longer than wide, asymmetrical, dark blue, blackish or brown. The mussel's external shell comprises two hinged valves, which are joined by a ligament and can close when necessary, by internal muscles (Dailianis 2011). Valves are lined by mantle skirts (left and right). In between is a mantle cavity filled with seawater. They have an organ called the foot, which is in marine mussels tongue-like shape. Mussels have filibranch gills formed by combined filaments. The gills divide the mantle cavity into ventral inhalant and dorsal exhalant spaces. Water enters the ventral edge of the shell and passes between gill filaments. Water flows from the ventral inhalant ship on, proceeds posteriorly and dorsally and finally flows back into the sea through the dorsal exhalant siphon (Dailianis 2011). Despite their respiratory function, gills also have a feeding function. Food passes through the short oesophagus and into the stomach (digestive gland system) (Dailianis 2011).

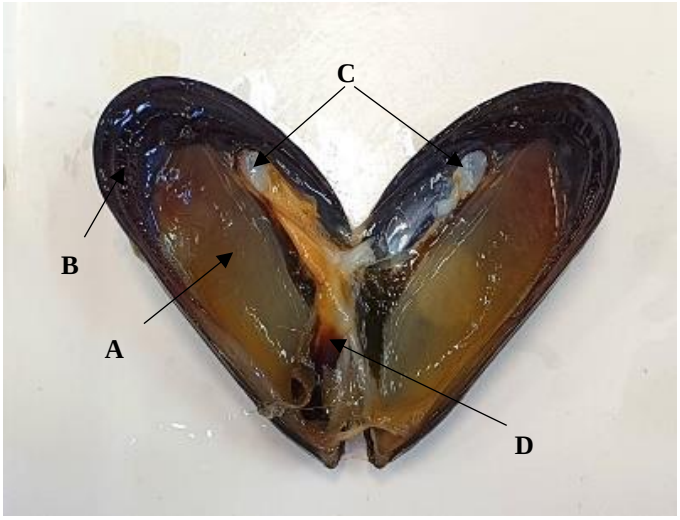

Figure 2: *Mytilus galloprovincialis* anatomy: A - gills, B - mantle, C - posterior adductor muscle, D – foot.

Mussels, like all bivalves, are suspension-feeding organisms, which can result in some particle reduction in the surrounding water (Cranford 2019). Bivalves process larger volumes of water per unit of time and can capture particles as small as 3µm with high efficiency (Ward et al. 2019). Based on literature, *Mytilus* spp. It is a promising bioindicator for the microplastic particles in the water column due to their ecology, availability, adequate sampling, sample processing, and further analysis (Lusher et al. 2017). Mussels are considered ecosystem engineers and foundation species that feed on microalgae, affect water turbidity, provide habitat heterogeneity and are essential aquaculture organisms. They are also known as organisms that ingest MPs in their natural habitats, while their clearance rate is sensitive to stress, making them one of the best biological indicators in polluted environments (Harris and Carrington 2019). The primary mechanism by which mussels

withdraw MP particles from the water column is filtration feeding by moving lateral-frontal gills cilia and particle retention (ingestion/egestion) in the mussel (Rissgard et al. 2014).

A comprehensive understanding of the biology and ecology of *Mytilus* sp. allows them to be used as bioindicators or as a tool for biomonitoring microplastics in the environment. The use of bivalves for biomonitoring purposes is not a new idea, and a study by Bendell et al. (2020) suggests that a good biomonitor should be globally distributed, abundant and its response to the contaminant of interest should be indicative of trends. For example, a study by Avio et al. (2015) provided evidence that MPs absorb PAHs, which elevates the bioavailability of these chemicals after ingestion. Therefore, they proved that *Mytilus galloprovincialis* had cellular responses, including immunological responses, neurotic effects, and genotoxicity.

A study by Brate et al. (2018) investigated the potential suitability of *Mytilus* spp. as sentinels for monitoring in coastal waters. They collected samples from various sites spanning the whole Norwegian coastline and found MPs in all locations. Zhao et al. (2018) research indicated that 70% of all sampled aggregates of *Mytilus edulis* (blue mussel) retained MPs and, over 40% of the MPs particles were rejected as pseudo-faeces or ingested in faeces. This suggests that mussel aggregates are essential in removing MPs from the ocean. In the study of Bendell et al. (2020), it is also stated that uptake, elimination, and accumulation of micro fragments by blue mussels (*Mytilus edulis*) were studied and that 71% of the fibers were quickly egested as pseudo-faeces with only 9% being ingested. A study by Hatoro et al. (2019) stated that in addition to selective feeding and rejection behaviour, bivalves, when transferred into fresh water, can depurate the contaminants from their inside, including MPs. In addition to nutrient particles, mussels filtrate MP particles, which are then ingested, accumulated, and egested as faeces and/or expelled as pseudo-faeces, which both naturally sink and deposit on the sea bottom, in addition to preventing the further spatial spreading of MPs pollution (Hamer et al. 2004; Hamer et al. 2008; Kanduč et al. 2011, Kanduč et al. 2018).

Therefore, *Mytilus galloprovincialis* is the predominant mariculture species of the *Mytilus* genus along the Mediterranean Sea. In Slovenia, *Mytilus galloprovincialis* is the primary shellfish species cultivated, making up 83% of the country's total mariculture output (FAO 2024). The key shellfish species farmed in Croatia include the *Mytilus galloprovincialis* and the European flat oyster (*Ostrea edulis*). In 2022, the total shellfish production amounted to 1,096 tonnes, with 1,006 tonnes attributed to *Mytilus galloprovincialis* (Eurofish International Organisation 2023).

Given the above facts, we decided that *Mytilus galloprovincialis* is the most suitable species to investigate MP biomonitoring properties purposes for this thesis.

Figure 3: The Mediterranean mussel farm in Strunjan, Slovenia (foto: Luka Preložnik, Landscape Park Strunjan).

2. GOALS, AIMS AND HYPOTHESES

2.1 Goals

The main goal of this research was to investigate the potential of *Mytilus galloprovincialis* filtration function as a sustainable tool for water column microplastic purification in laboratory conditions. The main idea was to use effective mussel MP filtration and sedimentation properties of produced faeces and pseudo-faeces particles to collect and remove MP fibers from the water column. Therefore, in addition to laboratory testing, one of this research's main goals was to develop a standardised method/installation that could be used in the natural environment for *in situ* seawater column MP monitoring at various locations along the eastern Adriatic coast.

2.2 Aims and main research questions

The primary aim of this thesis was to demonstrate mussels' filtration and purification efficiency using model suspended solids and CBs microfibers.
The following research questions have been opened:

- How many MP fibers do mussels filtrate in different time periods from 0 h up to 360 h of exposure?
- How many MP fibers sediment spontaneously?
- Is there any time difference between spontaneous MP fiber sedimentation and mussel filtration?
- What is the retention time of MP fibers in the mussels after exposure to elution containing MP fibers?
- In which time frame (how many hours or days after the beginning of exposure) is the filtration the most effective and suitable for biomonitoring?
- Is there any difference between MP fiber filtration, smoked (used), and non-used CB filters?
- What is the general contribution of mussels to MP fibers removal from the water column (bioremediation) and other ecosystem services?

2.3 Hypotheses

The main hypotheses of this research are:

- H1: With the presence of mussels, microplastic fibers are sedimented faster from the water column compared to passive sedimentation.
- H2: We can use *Mytilus galloprovincialis* as a suitable tool for the purification and monitoring of microplastic in the water column.

3. METHODS AND MATERIALS

In this chapter, all methods and materials used for the purposes of this research will be described in detail.

3.1 Chemicals

Throughout the research, the following list of chemicals was used:

- betadine (10% povidone-iodine, ALKALOID-INT),
- distilled water (dH_2O),
- filtered natural seawater (northern Adriatic).

Microplastic fibers were obtained from used and non-used CB filters composed of cellulose acetate (golden tip, premium quality, deluxe white tipping, 250 filter tubes, produced by Gizeh GmbH Germany).

3.2 Model organism – Mediterranean mussel (*Mytilus galloprovincialis)*

As discussed in Chapter 1, we selected the Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) as a potential bioindicator and a sustainable tool for monitoring and purifying the water column. Given that *Mytilus galloprovincialis* is the most common species of the *Mytilus* genus in the Adriatic Sea and based on literature reviews of various species within the same genus, it was logical to investigate the intake of microplastics (MP) by *Mytilus galloprovincialis* under laboratory conditions and to assess the potential effects on a broader spatial scale. In further text, Mediterranean mussels will be referred to as “mussels”.

3.2.1 Sampling site

For this research, mussels were provided from mariculture facilities in Lim Bay, Croatia (Figure 4) in February and April 2021 (Φ 45º 08,212'N, λ 13º 42,778'E). Based on the research objectives and the costs and availability aspects, we concluded that using cultivated populations is more reasonable than using wildlife populations, and cultivated aggregation can be monitored more closely and efficiently.

Lim Bay is an 11 km long, semi-enclosed marine inlet (channel-fjord like bay) of the Adriatic Sea on the west coast of Istria, 5 km north of Rovinj, Croatia. Due to its isolated geographical position, this inlet represents a unique environment that has favoured the development of endemic species of marine flora and fauna as well as shellfish farming since ancient times. Lim Bay has been a marine protected area – a special underwater reserve since 1967 (Pavičić-Hamer et al., 2016). Compared to the main coastal waters, Lim Bay exhibits variations and gradients in ecological parameters between different sites from east to west due to freshwater inflow (nutrients, temperature, oxygen content, salinity and water current velocity), which is responsible for habitat diversity (Hamer et al., 2010). Mussels (*Mytilus galloprovincialis*) are cultured in Lim Bay on ropes attached to rafts supported by plastic floats. The mussel ropes were suspended from the carriers, and each raft contained about 30 rope nets (with mussels) which were 2 - 4 meters long.

Figure 4: Sampling site, aquaculture facilities Lim Bay, Croatia. Upper left: The Istrian Peninsula located on the Adriatic Sea.

3.2.2 Acclimation and laboratory conditions

Mussels were transferred from the aquaculture facility to outdoor stone pools at the Centre for Marine Research Rovinj (Institute Ruđer Bošković, Croatia). They were placed in nets and exposed to a low to moderate, yet constant, seawater flow. The seawater temperature in the stone pools varied with environmental conditions and was not regulated. The mussels were not fed, as the seawater was unfiltered.

For each experiment, we selected mussels of approximately the same size (5-6 cm) and removed any encrusting organisms from their shells using a sharp knife. This was done prior to the commencement of the experiment to prevent any interference with the results.

3.2.3 Condition Index (CI)

The Condition Index is a significant health indicator that can be used to assess the impact of pollutants on mussel health. Consequently, it is valuable for environmental monitoring and aquaculture management. The higher proportion of the meat indicates better mussel health (Župan and Šarić 2014).

The shells' length, width, and height (Figure 5) were measured using a 0.05 mm precision calliper. Next, we obtained each mussel's total (live) weight (LW). Afterwards, we removed soft tissues from the shells and weighed the total meat weight (WMW) and shell weight (SW).

The CI data, a cornerstone of our research, were essential for ensuring the reliability and comparability between the data sets. Literature provides various equations to calculate the Condition index in mussels, the common factor among all of these is the proportion of the mussel's meat and its total weight and/or size (Pavičić-Hamer et al. 2016, Kanduč et al.2018).

For this thesis, we calculated CI (Meat yield) with the following equation:

$$CI\ (MY) = (WMW / LW) * 100$$

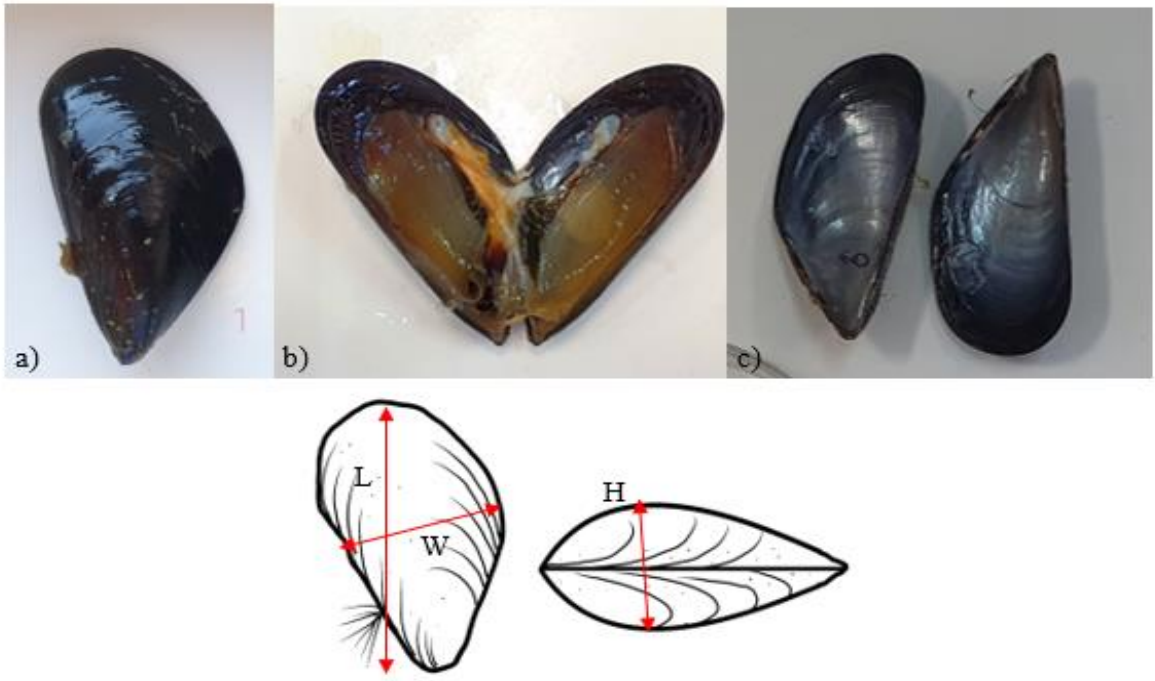

Figure 5: Condition Index measurements in *Mytilus galloprovincialis* were conducted for all experiment specimens. a) closed mussel, b) opened mussel, c) shells, L – length, W – width, H – height**.**

3.2.4 Gender determination

The environment's temperature influences mussel gametogenesis. Extreme changes can elicit a seasonal reproduction (Fearman and Moltschaniwskyj 2009).

For gender determination, we used mechanical stimulation and low temperatures to induce spawning. First, mussels were exposed to seawater at 5 °C for 5 min and transferred back to the seawater to which they had been previously acclimatized (15 °C). The cycle was repeated twice, and afterwards, the mussels were carefully opened, and a plastic stick (the thickness of a toothpick) was inserted between the shells (Figure 6) so the mussels could not close. Next, each mussel was placed in its bowl, into which we added fresh, filtered seawater so that the surface reached half of the mussel.

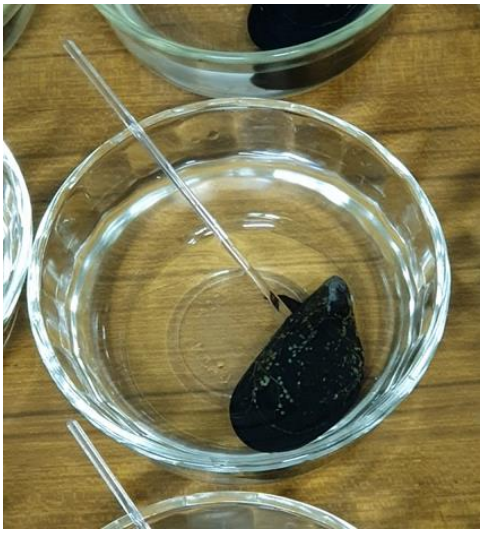

Figure 6: Induced mussels spawning.

Figure 7: Color and consistency difference between female (left) eggs and male (right) sperm cells.

After 30 min, we collected the samples, and the gender was determined based on the colour and consistency of the spawn and confirmed under the microscope (Figure 7).

3.3 Model microplastic

As described in the first chapter of the thesis, used CB are the most common beach litter, which was the primary reason for selecting them as the model source of MP.

3.3.1 Preparation of CB fibers exposure solution

Used and non-used CB filters composed of cellulose acetate (golden tip, premium quality, deluxe white tipping, 250 filter tubes, produced by Gizeh GmbH Germany) were used. The same protocol prepared the exposure solution for all the experiments included in this research.

CB filters were weighted and cut into 0.5 mm thick discs with a brand-new razor blade and then cut into smaller pieces with scissors. In 0.5 L of non-filtered seawater, approximately 11 mg (or two rings) of CB filters were added. Filters were blended for 3 minutes with a hand blender. After blending, we added another 0.5 L of non-filtered seawater (Figure 8). The total volume of the CB filter solution at the end was 1 L. We followed the same procedure for used and non-used CB filters.

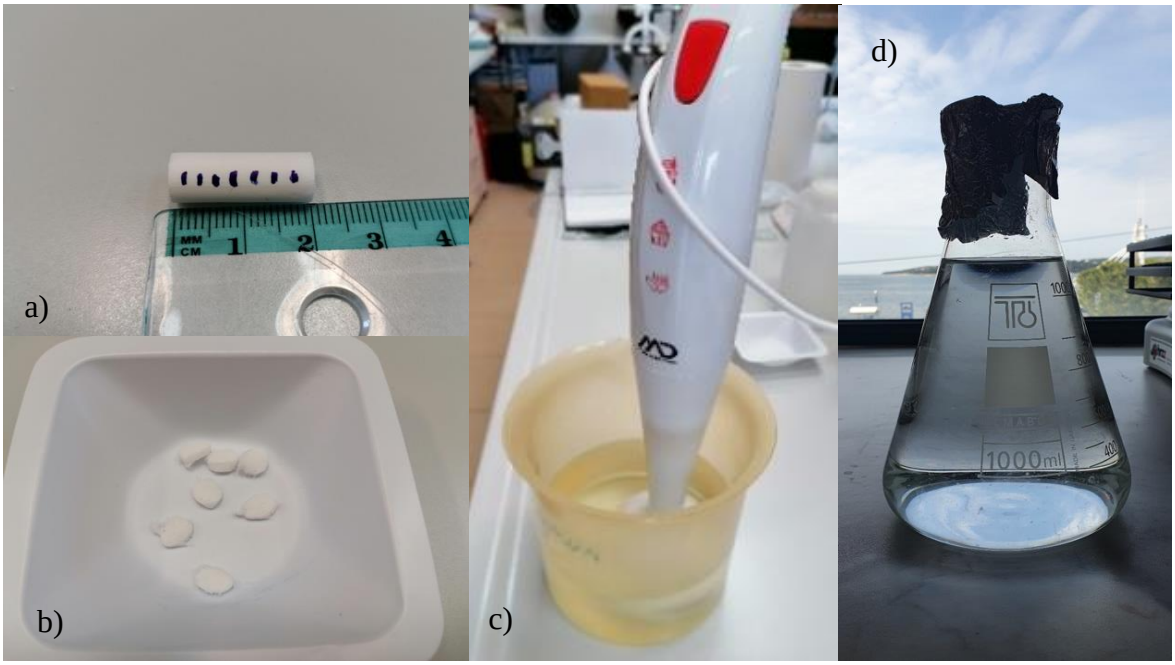

Figure 8: Preparation of microplastic solution. a) Standard cigarette butt, b) 0.5 mm discs, c) Blending CB in seawater, d) Prepared 1 L solution of CB MP fibers for the experiment.

3.3.2 Sample collection and preparation for visual MP fibers counting under microscope

CB microplastic fibers were counted in the water column, faecal pellets (with pseudo-faeces) samples, and the mussel's interior. The same protocol was used for all experiments conducted in this thesis.

For water column sampling, we used a 15 ml glass pipette (Figure 9). We took 10 ml of water three times for each sample and transferred it to a 15 ml falcon tube, where each sample was well mixed in a vortex before transferring on a six-well microplate, 5 ml of sample per well.

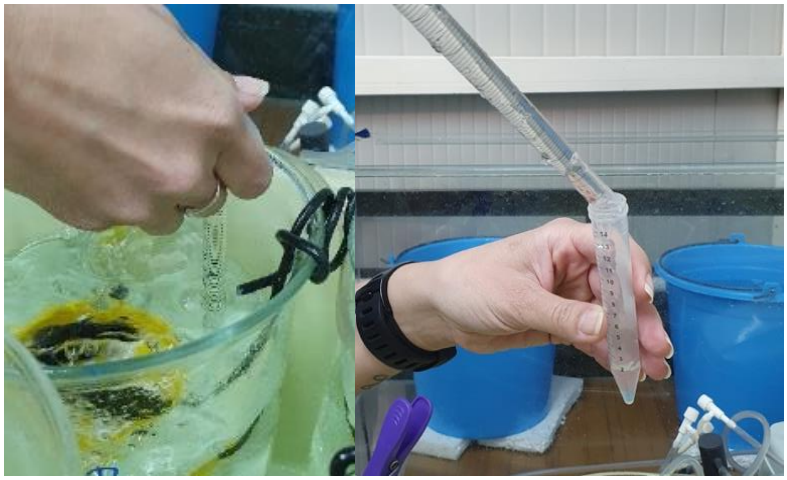

Figure 9: Water column sample collecting, for MP fibers counting.

Faecal pellet (including pseudo-faeces) samples were collected in 5 ml falcon tubes attached under the funnel with mussels (Figure 10). Before counting, samples were centrifuged at 4000 r/min for 5 min. Excessive water was removed with a micropipette till a total volume of 5 ml. Visually remaining byssus threads were removed with tweezers. Samples were then manually homogenized and mixed in the vortex for 1 min. We took 1.25 ml of prepared solution in the next step and transferred it to a 20 ml falcon tube, adding another 3.75 ml of distilled water (Figure 11). The prepared solution was well mixed in a vortex and transferred on a six-well microplate before counting. The method was used for faecal pellet sample preparation throughout the experiment, sedimentation samples in groups D and DU, and water column filtrates at the end of the experiment.

Figure 10**:** Faecal collecting method with a funnel and falcon tube.

Figure 11: Faecal samples prepared for MP fibers counting.

3.3.3 MP fibers counting

Before counting, a drop of Betadine (10% povidone-iodine, ALKALOID-INT) was added to each microplate well. Betadine pushed all remaining floating particles into the bottom of the well, making visual counting under a microscope easier.

Microplastic fibers were visually counted under an optical microscope (Zeiss Primovert Cell and Tissue Culture Microscope and Nikon Japan 811366 microscope) with 100x magnification. Samples were always counted in triplicates.

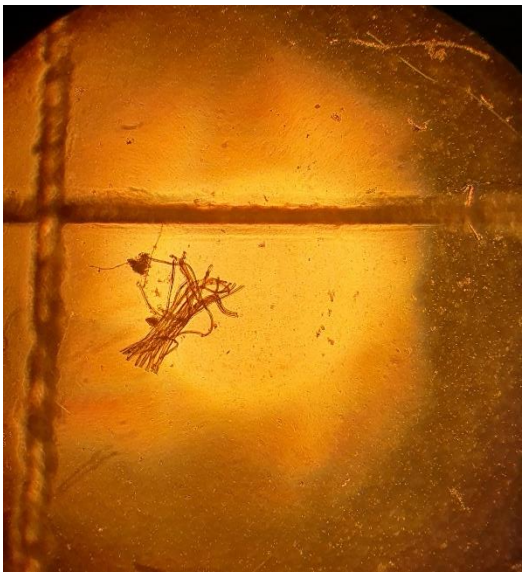

Figure 12: A cluster of MP fibers under 100x magnification.

3.3.4 Interior examination

After each experiment with MP fibers, the mussels were opened, and their interior was examined. The aim was to count microplastic fibers that could remain inside the mussel. Gills, internal byssus, gonads, and foot were separated and transferred on microscope slides, while inside water was transferred on the glass petri dish directly. The stomach of each mussel was checked for the content, and if the stomach was still full, we homogenized the tissue mechanically (Heidolph Hei-TORQUE CORE) for 7 minutes, with speed set at 1250. Samples were transferred on a microscope slide and counted at 100x magnification (same protocol as described in chapter 3.2.3).

Figure 13: Dissected mussel, prepared for internal examination.
Top left shows the shells; top right shows digestive system collected and ready for homogenization; bottom left shows the mantle and bottom right shows gills and the foot.

3.4 Filtration Rate Assessment

Filtration or pumping rate is defined as the volume of water flowing through the gills per unit of time. The clearance rate is the volume of water completely cleared of particles per unit of time. When all particles presented to the gills are removed from suspension, the clearance rate equals the filtration rate (Gosling, 2003).

Before assessing the microplastic fiber filtration efficiency of Mediterranean mussels, we concluded that it was necessary to determine the filtration rate of the mussels under laboratory conditions. The aim was to compare the sedimentation rate of organic matter mixed with MP fibers in the presence of mussels versus spontaneous sedimentation (without

mussels). We conducted a filtration rate assessment based on changes in turbidity, measured by increasing the luminance per unit area (lux).

First, we prepared a solution containing suspended organic matter (originating from a fish aquarium filter) with a total volume of 10 L. The experiment utilized two water tanks, each with a volume of 40 L. The control group was designated as Tank A, and the experimental group as Tank B. In Tank A, we added 15 L of fresh seawater and 5 L of the pre-prepared solution with suspended organic matter. In Tank B, we added 15 L of fresh seawater, 5 L of the pre-prepared solution with suspended organic matter, and 20 mussels.

Tanks A and B were placed on a flat surface. A table lamp was installed between the tanks as a light source. Sensors positioned on the opposite side of the tanks measured changes in turbidity by detecting changes in light (lux). The sensors were set to sample at 5-minute intervals. The water temperature was constant (17 °C). Data was collected after the 120 minute experiment. Figure 14 shows a graphical representation of the experiment. At the end of the experiment, we measured the condition index in a sample of 5 randomly chosen mussels.

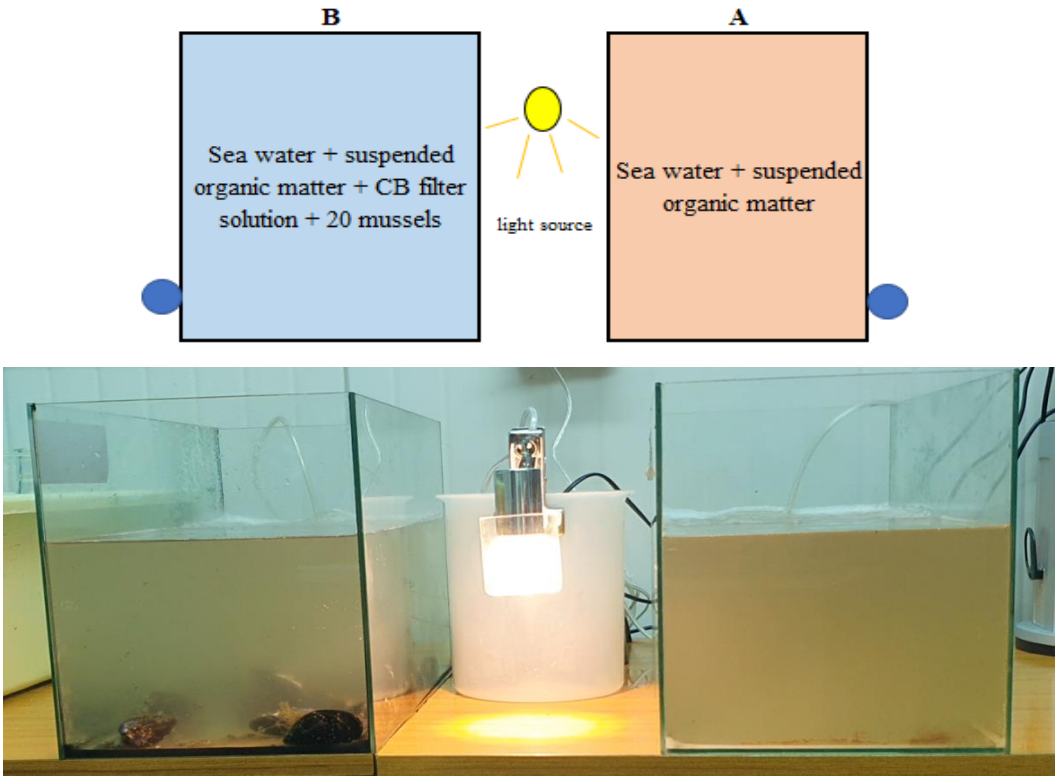

Figure 14: Experiment design for filtration rate assessment.

3.5 Filtration and Sedimentation Rate of MP Assessment

For filtration and sedimentation rate assessment of MP fibers in the presence of mussels, we used non-used cigarette butts (CB) and used cigarette butts (CBU). MP fiber solution for non-used (CB) and used (CBU) was prepared by the same protocol described in Chapter 3.2.1.

3.5.1 Experiment design

The experiment consisted of 2 major groups, divided into CB and CBU categories. Each group was then divided into one control group with mussels without MP fibers (marked as A), one control group with MP fibers and without mussels (marked as D), and two groups consisting of MP fibers and mussels (marked as B and C).

As described in Chapter 3.2.2, we collected samples of faeces and pseudo-faeces. For this, we placed two mussels in a plastic funnel, under which we attached a 5 ml falcon tube. All beakers containing mussels had the exact positioning and set-up.

Group A and AU contained two mussels and 4.5 L of fresh and filtered seawater.
Groups B, BU, C, and CU contained two mussels, 5 L of fresh and filtered seawater, and 1 L of CB or CBU solution. Groups D and DU comprised 3.5 L of fresh seawater and 1 L of CB or CBU solution (Figure 15). In each beaker, constant aeration was set.

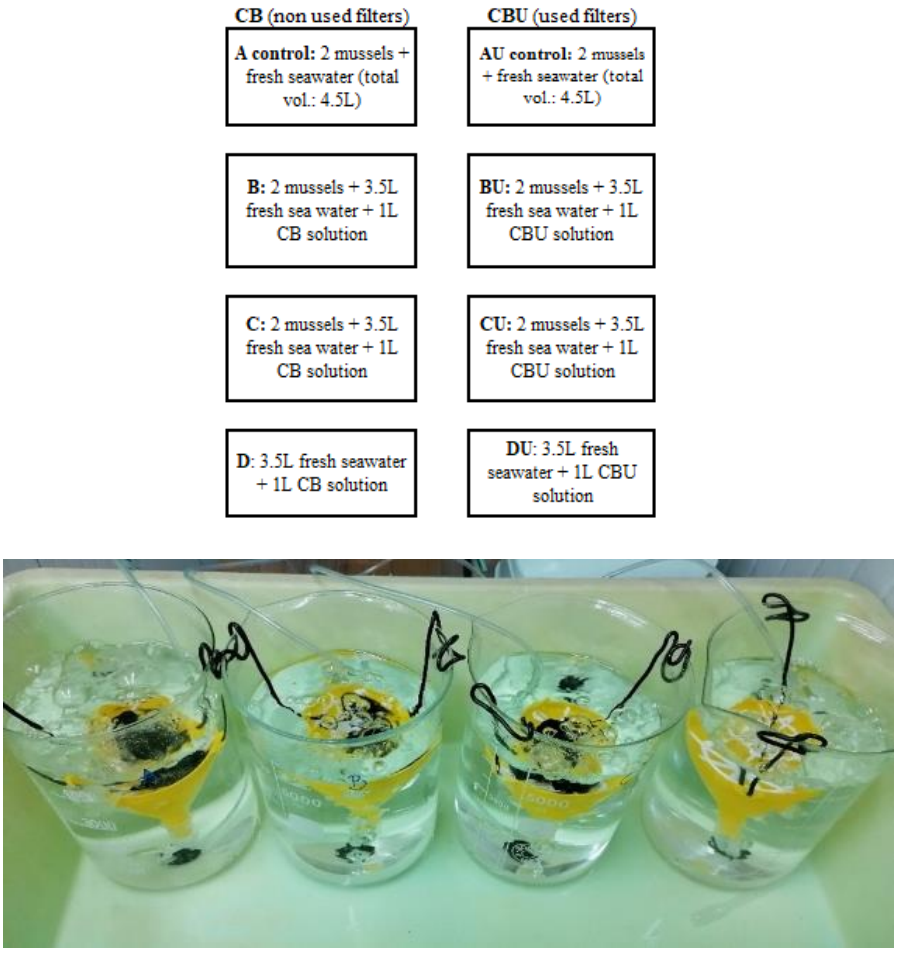

Figure 15: Experiment design for filtration and sedimentation rate assessment.

During the experiment, mussels were fed with 20 ml of *Tetrasemis chuii* and *Chlorella* sp., every 24 h.

3.5.2 MP fibers samples collecting and preparation

Mussels were exposed to microplastic fibers for 360 hours (15 days). On the first day, we collected samples every 3 hours (at intervals of 0h, 2h, 6h, 9h, and 12h). In the following days, we collected samples every 24 hours. The protocol for sample collection and MP fibers counting is described in Chapter 3.2.2.

3.6 Mussel Purification Rate Assessment

This experiment aimed to assess the purification rate of the mussels after different exposures to the microplastic fibers. For purposes of this experiment, we used non-used cigarette filters. The protocol of the initial solution with CB fibers was the same as described in Chapter 3.2.2, with an initial volume of 2 L. With sample counting (10 ml), we estimated that the exposure concentration is 2000 MP fibers per litre.

In this experiment, we exposed mussels to MP fibers in different time frames (0h – 7h) using the same set-up with 5 L beakers and funnels as in previous experiments. After the exposure to MP fibers, mussels were placed in a 40 L tank with fresh seawater and constant aeration. Samples with faecal and pseudo-faecal pellets were collected every 24 h for 5 days (Figure 16). During the experiment, mussels were fed with 20 ml of *Tetrasemis chuii*, every 24 h.

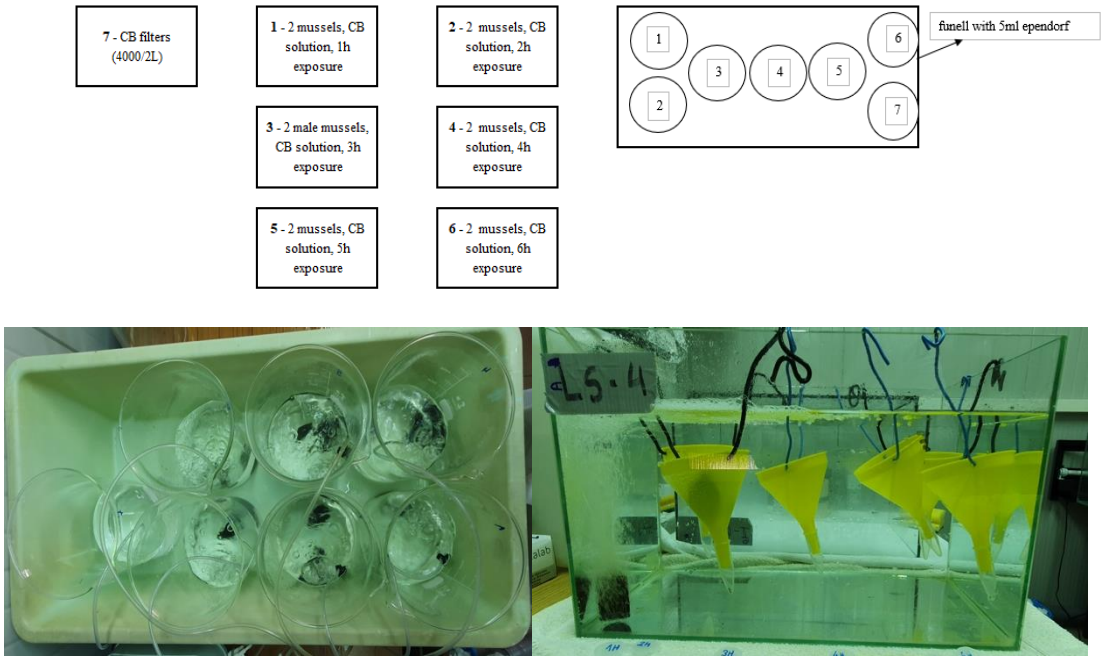

Figure 16: Experiment design for purification rate assessment.

3.7 Toxicological Assessment of Used CB

We chose the flaxseed germination test to assess the toxicological aspects of CBU. The first step was preparing the CBU solution, for which we used 1 CBU (0.1032 g) and 45 ml of dH_20. Then, the filter was cut into small pieces with scissors and placed in a falcon tube, which was placed on an orbital shaker for 24 h. The calculated concentration of the initial solution was 2.3 g/L.

From the initial CBU solution, we prepared 6 test solutions with the following CBU concentrations: 11.5 mg/L, 4.6 mg/L, 2.3 mg/L, 1.1 mg/L, 0.5 mg/L and 0.2 mg/L. To ensure the reliability of our results, we included a control group containing only dH_2O. Each testing solution had a volume of 5 ml, maintaining a consistent approach.

The test was conducted in triplicates. On each petri dish, we separated 3 groups of 30 flaxseeds (PrimaVita, 200g, Origin: Kazakhstan, purchased: 24. 5. 2021) on a layer of tissue paper. Afterwards, testing solutions were added to each petri dish, marked with a number indicating the volume of the initial CBU solution (Figure 17). All 7 Petri dishes were incubated at 25 °C for 24 h. After incubation, sprouted and non-sprouted seeds were counted.

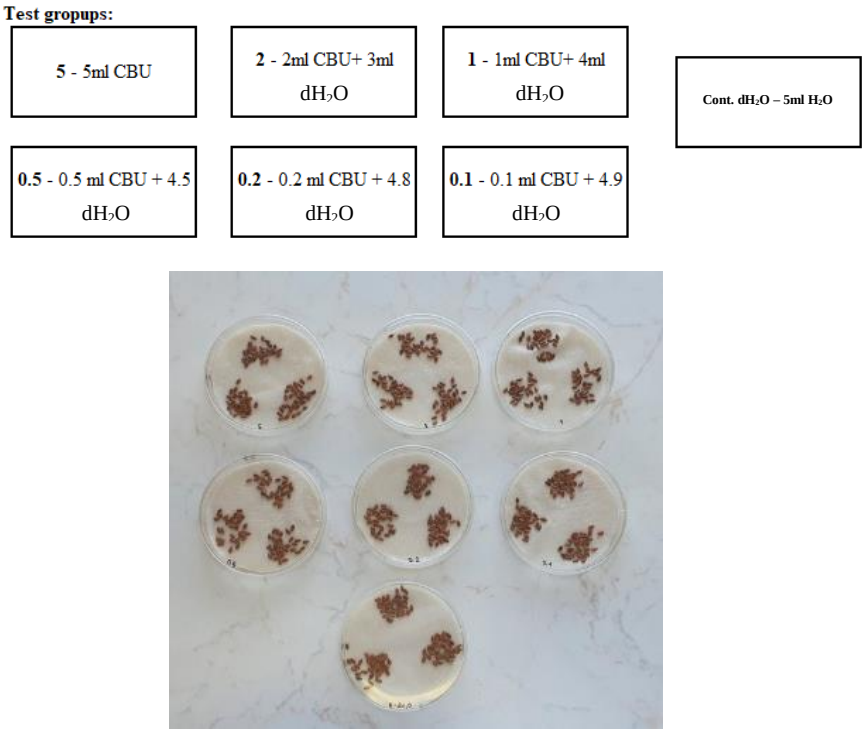

Figure 17: Experiment design of flaxseeds germination test, assessing the toxicological aspect of used CB.

3.8 Statistical analysis

For the data analysis purpose, we obtained various statistical analysis methods, including:

- Regression analysis,
- One–way ANOVA with post hoc Tukey and/or Bonferroni tests.

Statistical analysis was conducted in Microsoft Excel. Differences were considered significant when $p < 0.05$.

4. RESULTS

4.1 Filtration Rate Assessment

The results showed a difference in the change in light intensity between aquarium A (the control group) and aquarium B, which contained mussels. In aquarium B, where the mussels were present, the light intensity increased from 370 lux to 520 lux. The gradual increase in light intensity over time in presence of mussels was also confirmed by linear regression ($p < 0.05$). In contrast, the light intensity in aquarium A, which did not contain live mussels, remained unchanged during the measurement period which was confirmed with linear regression ($p > 0.05$) as well. Based on the results, we can infer that spontaneous sedimentation of organic matter occurs noticeably more slowly compared to when living mussels are present (Figure 18). The visual representation of the beginning and the end of the experiment is shown on Figure 19.

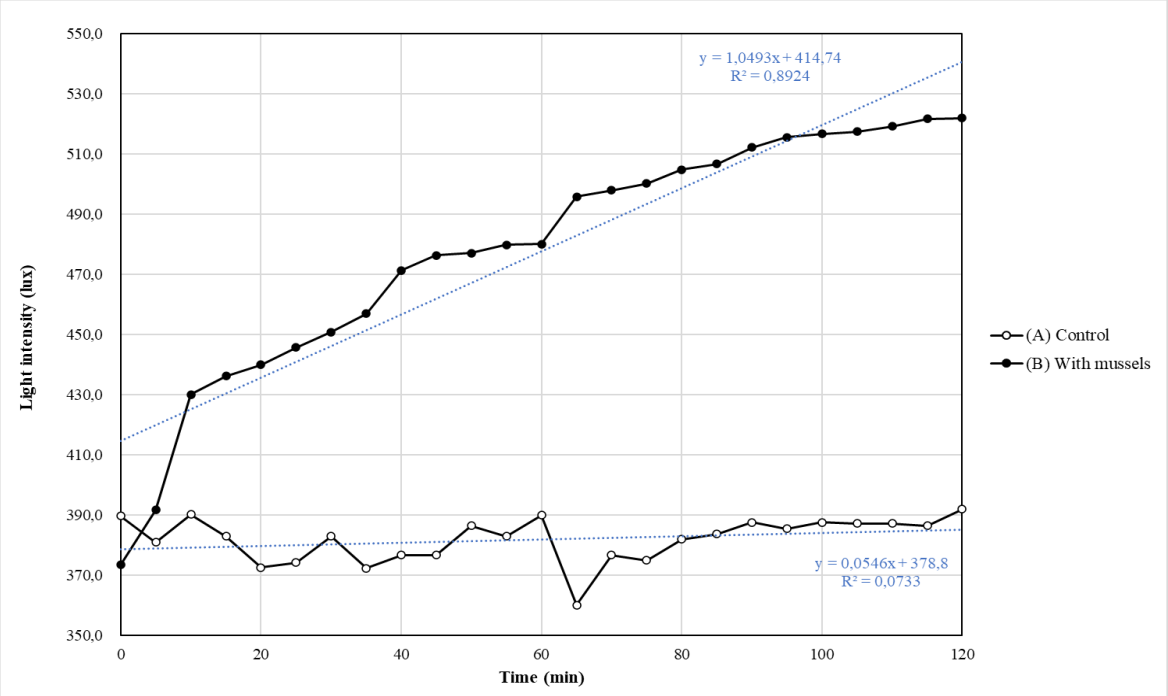

Figure 18: Data obtained from the filtration rate assessment, with linear functions added.
A) control without mussels B) with mussels; R linear regression.

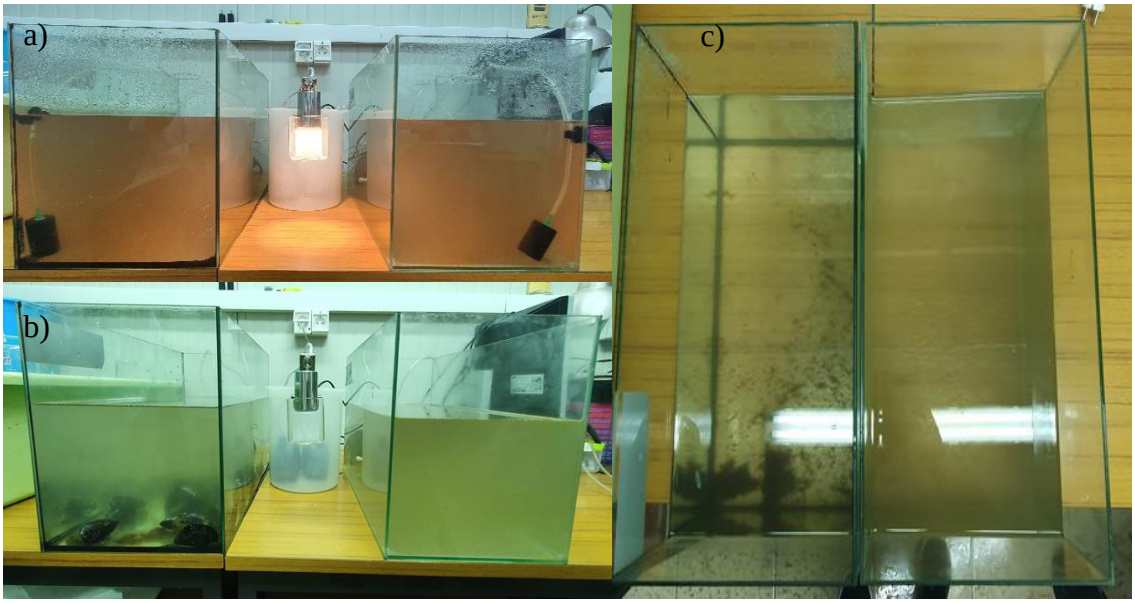

Figure 19: Visual representation of the filtration rate assessment's beginning (a, b) and end (c). On the right is Aquarium B with the mussels, on the left is Aquarium A without the mussels.

The average condition index in a sample of 5 mussels from the filtration rate experiment was 24.1, with a maximum value of 28.1 and a minimum value of 23.1. The condition index of each mussel in the experiment did not differ significantly from the average value; therefore, we can conclude that the mussels' filtration function was similar throughout the whole experiment, and the data can be generalized (Figure 20).

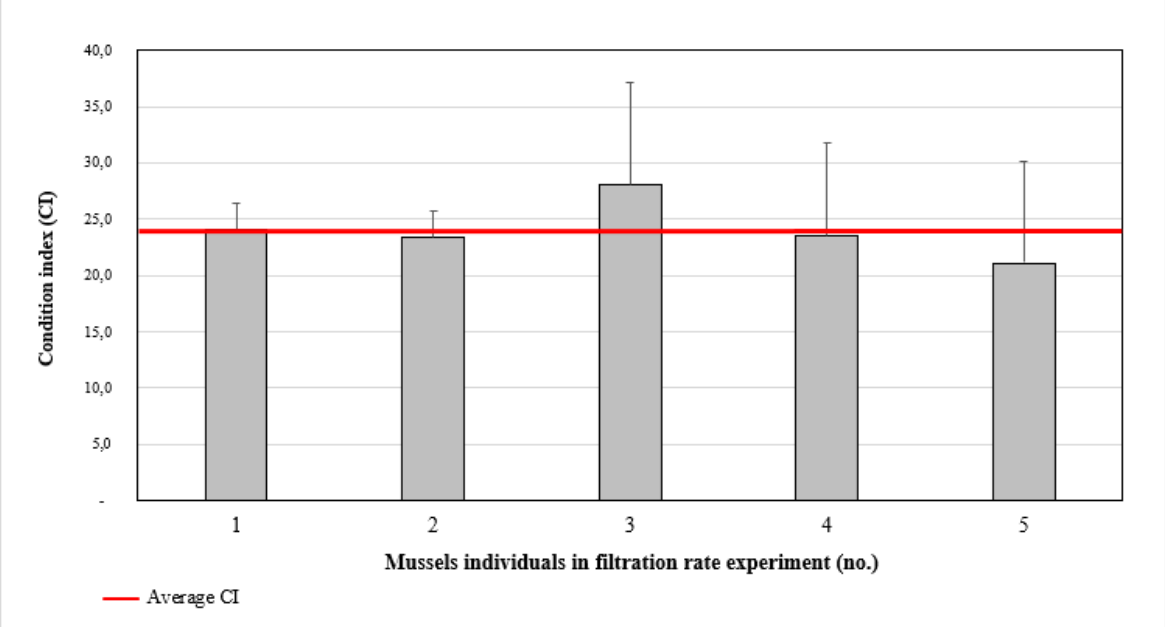

Figure 20**:** Condition index of each individual mussel in the filtration rate experiment with average value.

4.2 Filtration and Sedimentation Rate of MP Assessment

The data showed that the number of MP fibers in the water column decreased over time, regardless of whether active filtration was present or not. However, the results in graphs (Figures 21 and 22) indicate that the number of MP fibers decreases more rapidly when active filtration is present, dropping below 10% of the total number of fibers in first 10 h of the experiment and stayed constant throughout the whole time of the experiment. A similar trend was present in both testing groups (CB and CBU), from which we can conclude that the mussels incorporate MP fibers during the filtration process, regardless of whether the source of the fibers is a used or non-used CB.

When observing the number of non-used CB (Figure 20) MP fibers within the faecal pellet, mussels deposit approximately the same percentage of fibers into the faecal pellet as those that spontaneously deposited. The deposition into the faecal pellet through filtration was faster during the first 120 hours of the experiment.

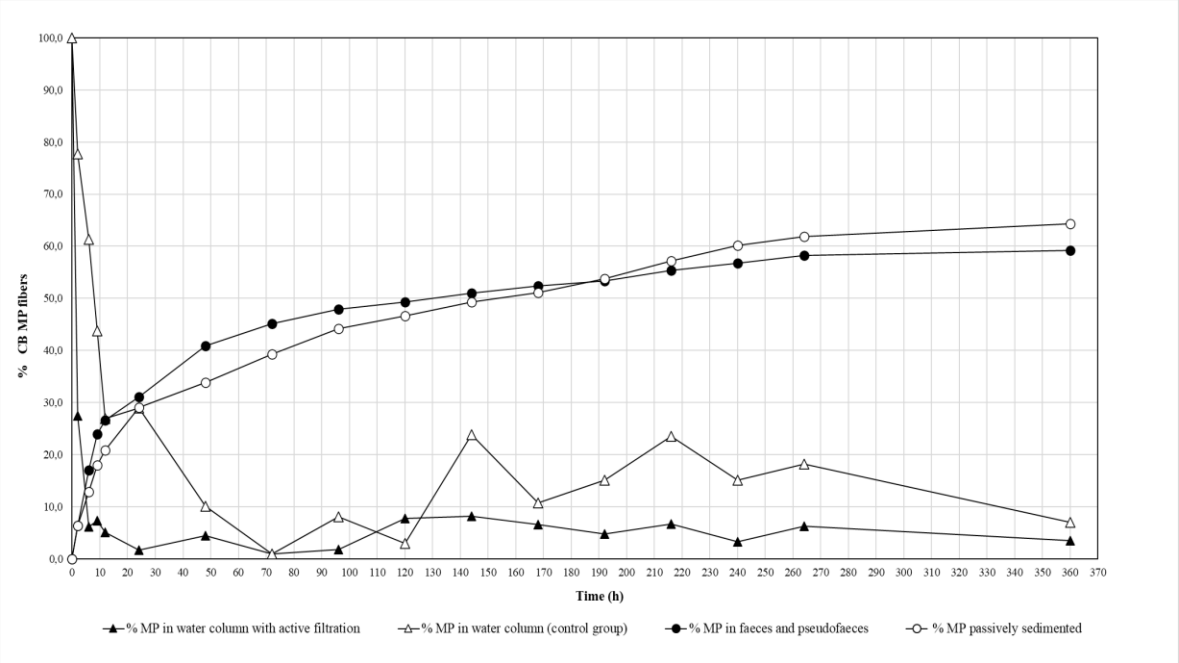

Figure 21: Percentage of non-used CB MP fibers through time, counted in the water column with active mussel filtration in a control group without mussels, in faecal pellet produced by mussels filtration and passive sedimentation, through the time of exposure to MP fibers.

The data from the group containing used CB (Figure 22) showed that mussels deposit fibers into the faecal pellet faster than the fibers can passively sediment, and they also deposit a higher percentage of fibers into the faecal pellet than the fibers can passively sediment. However, the higher amount of MP fibers passively sediment is shown in the faecal pellet.

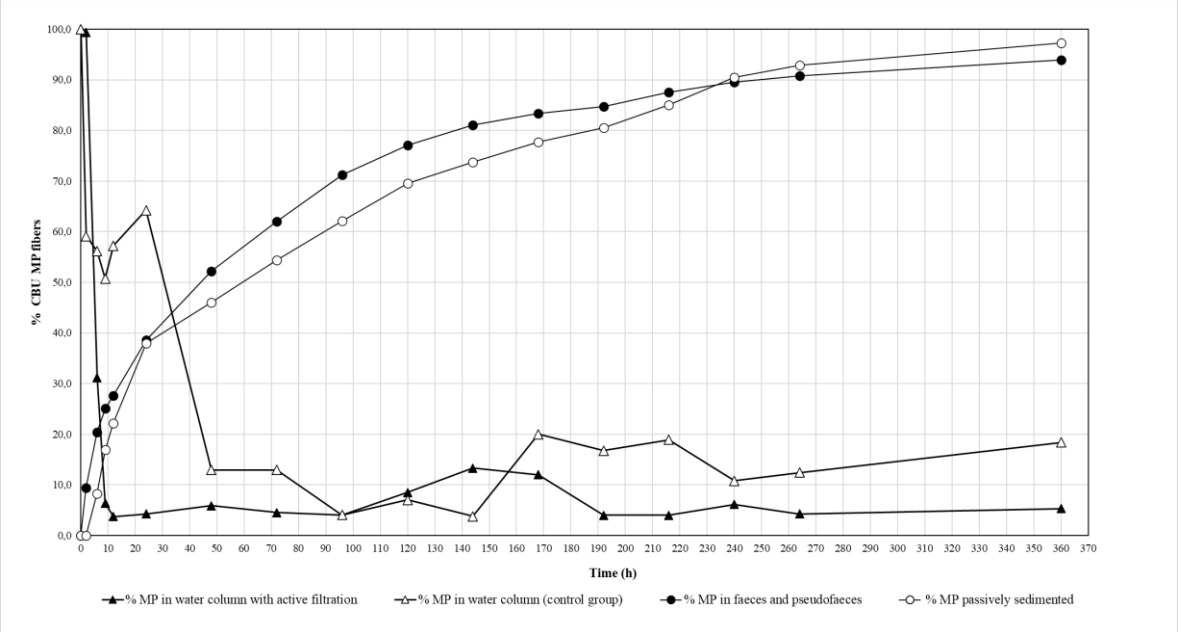

Figure 22: Percentage of CB MP fibers and CBU MP fibers in faecal and pseudo-faecal pellet through a time of exposure to MP fibers.

In summary, the data demonstrate that the percentage of MP fibers decreases more rapidly in the presence of mussels. The percentage of fibers that spontaneously sediment is approximately equal to the percentage filtered by the mussels. However, it cannot be overlooked that the removal of fibers from the water column occurs more quickly in the presence of mussels than without them, regardless of whether the fibers come from used or unused cigarette butts.

When comparing the percentage of used and non-used CB fibers found in the faecal pellet of the mussels, it is evident that fibers obtained from used cigarette butts accumulate in the faecal pellet faster and in more significant quantities compared to fibers obtained from unused cigarette butts (Figure 23).

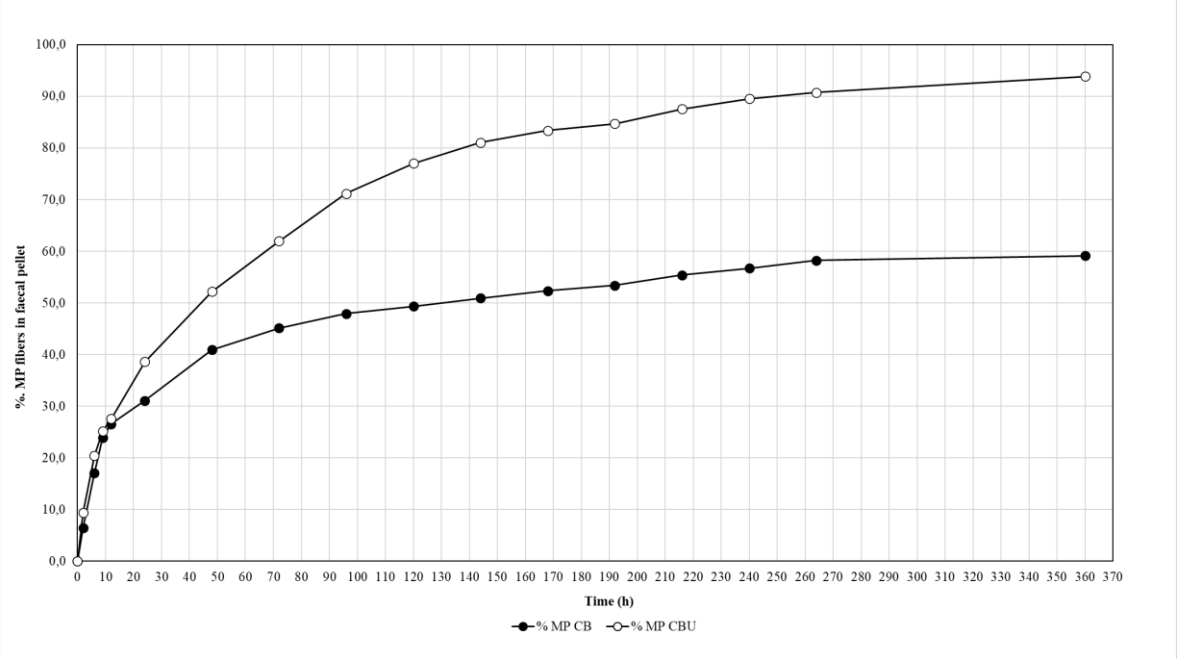

Figure 23: The percentage of CB and CBU MP fibers found in the faecal pellet of mussels through a time of exposure to MP fibers.

Assuming that mussels are living organisms that respond to environmental changes, the data suggest that mussels react to the presence of toxins by attempting to expel them from their bodies as quickly and in as large a quantity as possible.

From the observed data, we can conclude that CBU fibers have a greater tendency for passive sedimentation, most likely due to the presence of additional chemical compounds that accumulate in the cigarette filter after smoking and originate from the tobacco section, shown in Figure 24.

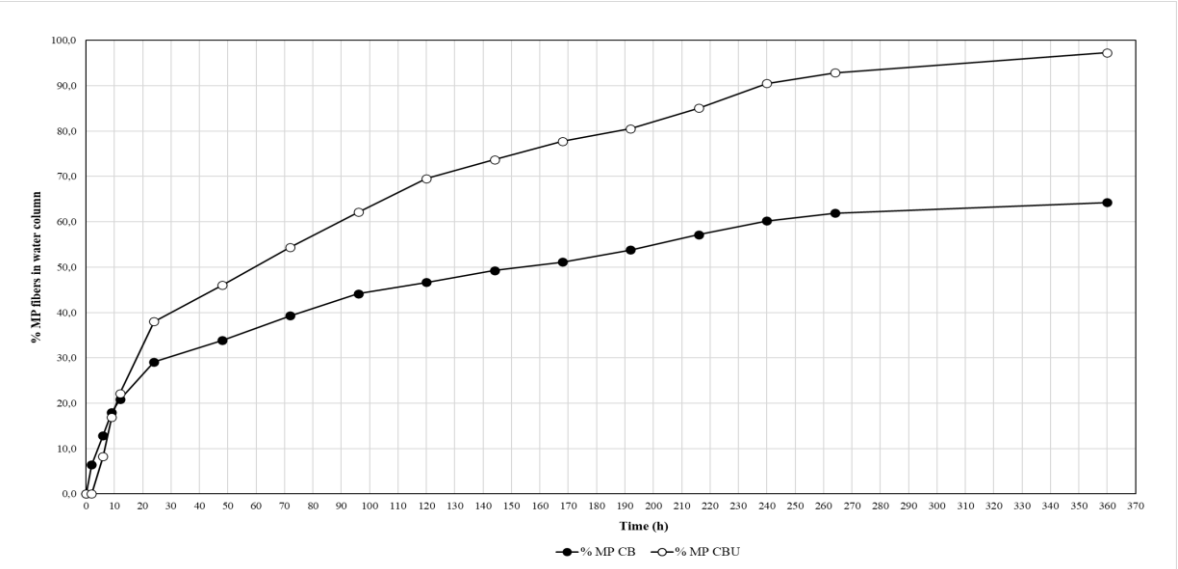

Figure 24: Percentage of CB and CBU MP fibers which passively sedimented through time.

At the end of the 15-day experiment, we dissected the mussels and examined their interiors to detect any potential microplastics on the surfaces of their internal organs. The results indicated that 0.1% of an initial number of MP fibers were present on the surfaces of the internal organs of the mussels which were exposed to CB MP fibers, and 0% of fibers were found in the interior of the mussels which were exposed to CBU MP fibers. Additionally, 0.96% of all MP fibers were found in the water column after the experiment in the group where CB MP fibers were applied, and 0.2% in the water column where CBU MP fibers were applied, leading us to assume that the mussel actually ingests the majority of fibers through the digestive tract and subsequently expelled through faecal and pseudo-faecal pellet. Figure 25 presents the percentage of MP fibers according to position where they were found.

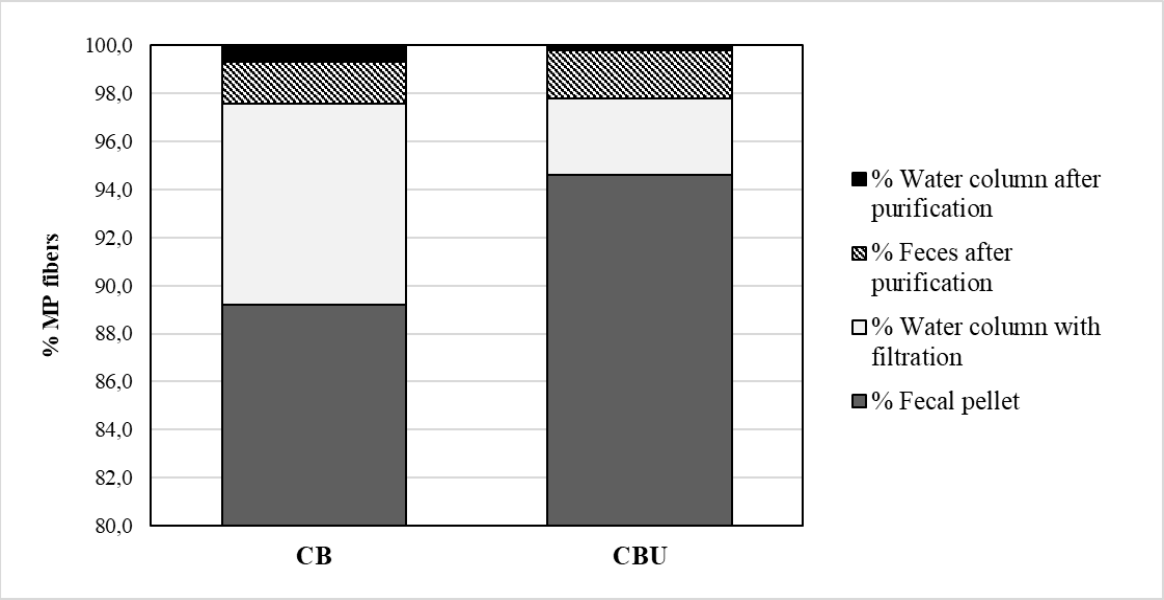

Figure 25: The percentage of MP fibres in sample groups is based on the location where they were found.

In conclusion, data showed that mussels expel between 85% and 90% of all MP fibers with faecal and pseudo-faecal pellet. The remain 10% of MP fibers were excreted trough faecal and pseudo-faecal pellet in the process of purification which lasted 6 days.

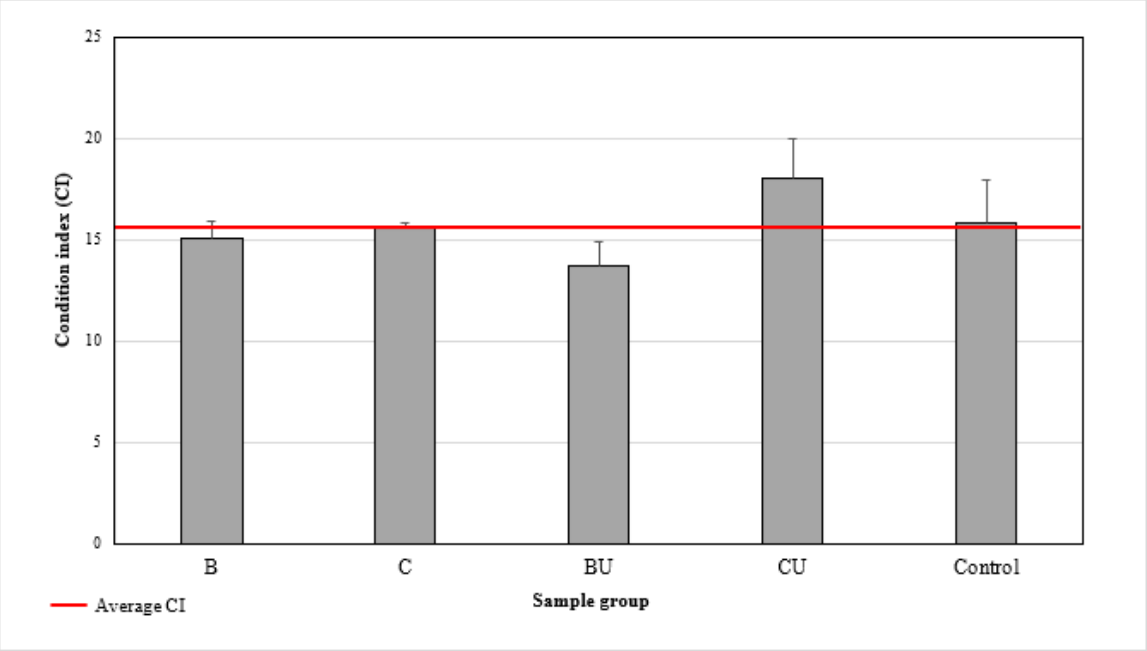

Figure 26: The Condition index of the mussels in the filtration and sedimentation rate experiment within the sample group B, C, BU, CU and Control.

The average condition index of the mussels used in this experiment was 15.6, with a maximum individual value of 1.4 and a minimum individual value of 12.9. The condition index of mussels was not statistically different between the sample groups (One-way ANOVA, $p > 0.05$). Therefore, we concluded that the results could be compared (Figure 26). Further analyses on purification rate were needed to get a better picture about time frame of collecting faeces and pseudo-faeces samples.

4.3 Purification Rate Assessment

The purification rate assessment results showed that the purification rate mussels need to exclude MP fibers from their system varies depending on how long they were exposed to the source of MP fibers and how long they were in a purification process (Figure 27).

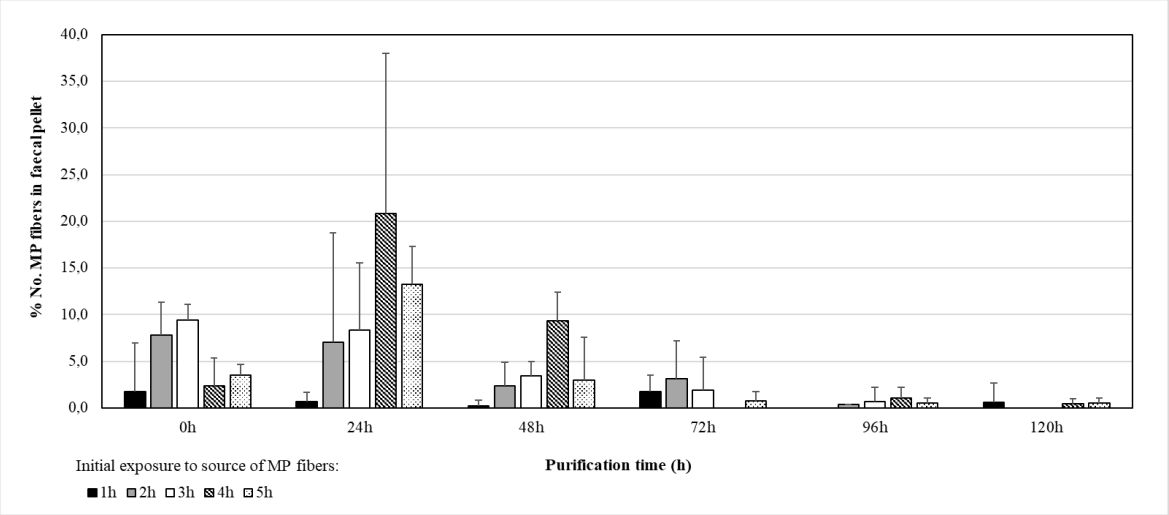

Figure 27: The percentage number of MP fibers found in the faecal pellet of the mussels through purification time (120 h) and within the initial exposure to MP fiber source duration.

Data shows that the most significant amount of MP fibers was expelled in the faecal pellet after 24 h of purification in mussels, regardless of the time of exposure to the source of MP fibers. If we compare the data between exposure times, it is noticeable that the most significant amount of MP fibers was observed in mussels exposed for 4 hours and the least in mussels exposed for 1 h.

The total amount of MP fibers (Figure 28) in faeces was the largest after 24 h of purification and then gradually decreased towards the end of the experiment (after 120 h).

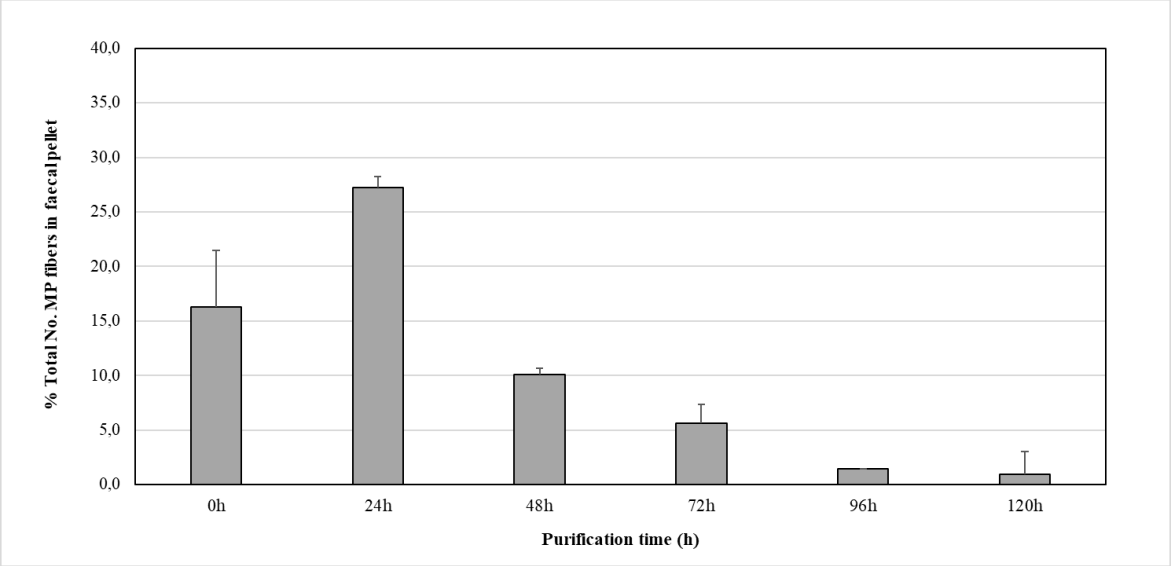

Figure 28: The percentage of the total amount of MP fibers in faecal pellet through purification time within all the exposure time groups.

In addition, data on MP fibres counting throughout the purification process showed a decreasing proportion of MP fibers in the water column right after exposure to the initial source of MP fibers and an increasing number of MP fibers in the faecal pellet right after exposure to the initial source of MP fibers and during the purification process.

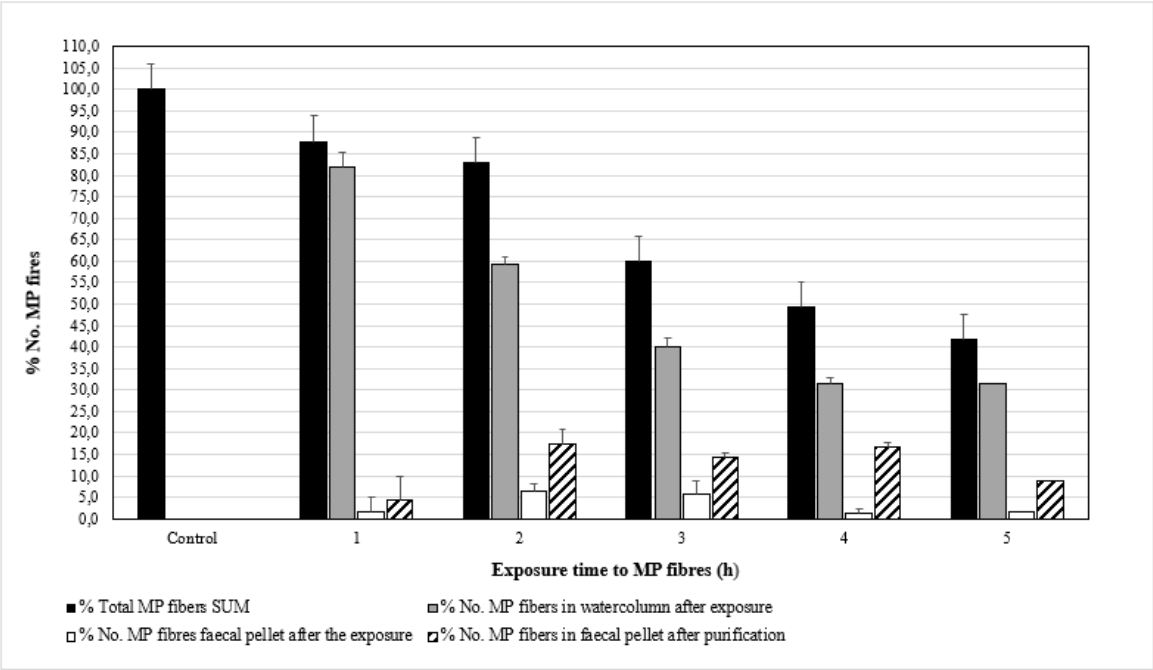

Figure 29: The percentage of MP fibers number within the exposure time to the initial source of MP fibers and disaggregated by the location where they were found.

In conclusion, a larger amount of MP fibers was evidently expelled from the mussel's interior in the span of 24 h of the purification process, which is 27% of all MP fibers in the initial exposure suspension, which was approximately 2000 MP fibers per litre (Figure 29).

4.4 Toxicological Assessment of Used CB

Figure 30 shows that a statistically significant difference in delayed germination of the flax seeds occurred between a control group which contained dH_2O and a test group containing a dilution with CBU concentration of 11.5 g/ml (One-way ANOVA, $p < 0.05$, confirmed with Tukey HSD test).

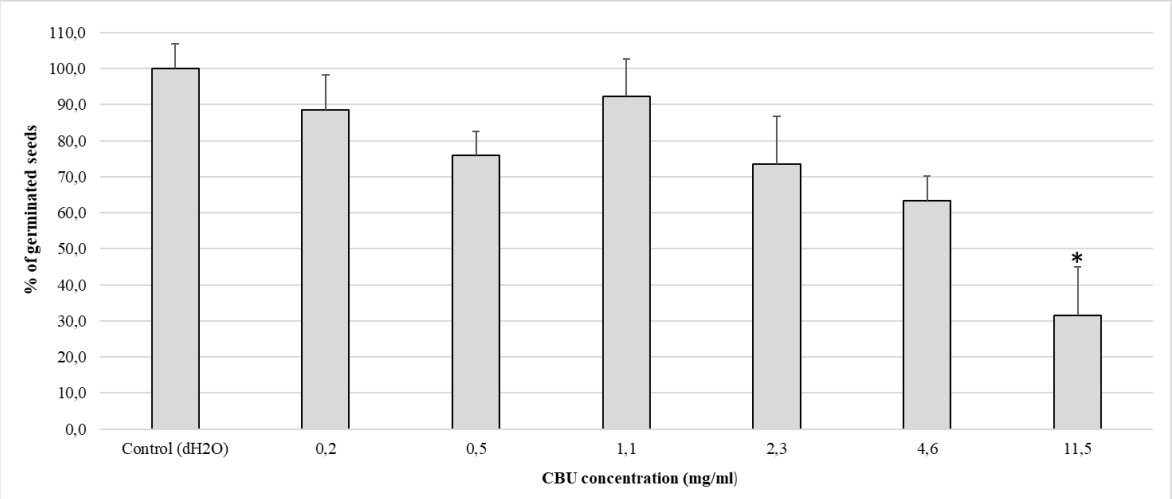

Figure 30: Average proportion of germinated flax seeds related to the CBU concentration (mg/ml).

From the collected data, we can conclude that the chemical compounds present in used cigarette butts, which were used for this thesis research, affect the potential germination of seeds and delay it. Although the flax seeds eventually did germinate, this might suggest that it would be reasonable to repeat the test in the future with a broader range of concentrations and on various organisms. For this thesis, however, further testing was not crucial, as the topic focuses on other aspects, and the potential toxicity of chemicals in cigarette butts is already well-researched according to the reviewed scientific literature.

5. DISCUSSION

Hidalgo-Ruz et al. (2012) proposed three general MP sampling methods in the marine environment. Samples can be selective, bulk or volume reduced. The article states that the volume-reduced method is the most commonly used in water column sampling, as the volume of the bulk sample is usually reduced during sampling. Research mentions that the water-reducing method is obtained with nets and filtering of large volumes of water, which can be time-consuming. Our research has significant implications, as we have demonstrated that *Mytilus galloprovincialis*, a commonly found bivalve, can serve as an effective biomonitoring organism for detecting MP fibers in the water column. This is due to its filter-feeding function and the fact that it excretes a majority of MP fibers through faeces and pseudo-faeces, effectively reducing the volume of MP in the marine environment.

The fact that *Mytilus galloprovincialis* is commonly found naturally and as a mariculture species in the eastern Adriatic Sea further reinforces their effectiveness as a biomonitoring organism in this region. (Hamer et al. 2004; Hamer et al. 2008; Kanduč et al. 2011, Kanduč et al. 2018). Aquaculture establishments have further practical use in MP fibers monitoring, as the *Mytilus galloprovincialis* growing method allows them to be actively present in the water column, enabling direct and continuous *in situ* water column MP monitoring. Laboratory results show that collecting the mussel's pseudo-faeces and faeces method approach is also suitable for more extensive use of coastal microplastic biomonitoring in combination with sea surface (Manta net) and sea bottom (sediments) analyses.

In general, the number of MP fibers in the water column of experimental aquaria decreased over time, regardless of whether active filtration was present or not. However, our results indicate that the number of MP fibers decreases more rapidly when active filtration (mussels) is present. A similar trend was also observed for both testing groups using model MP fibres (CB and CBU), from which we can conclude that the mussels incorporate MP fibers during the filtration process, regardless of whether the source of the fibers a used (smoked) or non-used CB (non-smoked) is.

When observing the number of non-used CB MP fibers within the faecal pellet, mussels deposit approximately the same percentage of fibers into the faecal pellet as those that spontaneously deposited during experiment time (120 h). However, deposition into the faecal pellet through filtration was faster and mainly occurred shortly after exposure 1, 6, and 12 h, as pseudo-faeces and 24-48 h as faeces.

Data shows that the most significant amount of MP fibers was expelled in the faecal pellet after 24 h of purification in mussels, regardless of the time of exposure to the source of MP fibers. If we compare the data between exposure times, it is noticeable that the most significant amount of MP fibers was observed in mussels exposed for 4 hours and the least in mussels exposed for 1 h.

From our results comparing CB and CBU fibre's passive sedimentation, we can summarize that CBU fibers have a greater tendency for passive sedimentation, and their distribution and spreading in the marine environment will be more local. In addition to that, we demonstrate that the percentage of MP fibers decreases more rapidly in the presence of mussels, and in field – real situations, it can be expected that besides disturbances of mussel filtration, additional effects of used-smoked cigarette butts (CBU) can occur.

From the collected data, we can conclude that the chemical compounds present in used cigarette butts, which were used for this thesis research, affect the potential germination of seeds and delay it. Although the flax seeds eventually did germinate, this might suggest that it would be reasonable to repeat the test in the future with a broader range of concentrations and on various organisms. For this thesis, however, further testing was not crucial, as the topic focuses on other aspects, and the potential toxicity of chemicals in cigarette butts is already well-researched (Micevska et al. 2006, Novotny et al. 2009). Cigarette butts are the most common form of litter, as an estimated 4.5 trillion cigarette butts are thrown away every year worldwide. Many chemical products are used during the course of growing tobacco and manufacturing cigarettes, the residues of which may be found in cigarettes prepared for consumption.

Additionally, over 4000 chemicals may also be introduced to the environment via cigarette particulate matter (tar) and mainstream smoke. The toxicity of cigarette butt leachate was found to increase from unsmoked cigarette filters (no tobacco) to smoked cigarette filters (no tobacco) to smoked cigarette butts (smoked filter + tobacco) (Slaughter et al. 2011). The toxicity of cigarette butts to marine organisms represents the potential ecological risks of cigarette butts to the aquatic environment. Wastewater treatment plants (WWTPs) are a focal point for the removal of microplastic (MP) particles before they are discharged into aquatic environments. WWTPs are capable of removing substantial quantities of larger MP particles but are inefficient in removing particles with any one dimension of less than 0.1 mm (Freeman et al. 2020).

Although one of the goals of this thesis was to investigate the potential method of MP fibers counting, which would be practical, precise and time-effective, therefore we also examined various methods of water column sampling. We wanted to verify whether our microscope counting was sufficiently accurate by first sampling the number of fibers in the initial suspension using two methods and comparing them. Then, we applied the calculated average value to the counting data of individual locations of MP fibers from the experiment. Data showed that the total amount of MP fibers found through the experiment in the mussel's interior, faecal pellet, and water column decreased over time, despite the initial assumption that at least the majority of MP fibers should have been count.

The counting was done in triplicate, with a 10 ml sample each taken from the entire 2 L sample. The number of counted fibers was then mathematically extrapolated to a 2 L sample in order to optimize the counting time relative to the number of samples we had with the initial assumption that the MP fibers were evenly distributed throughout the suspension, which was primarily achieved through the method used to prepare the initial suspension.

Upon closer examination, we found that our counting method has limitations. Specifically, we observed that MP fibers tend to clump together, especially in samples with a higher concentration of MP fibers. This can significantly affect our method's passive sedimentation rate and counting accuracy, making mathematical extrapolation from a smaller sample to a larger one less accurate.

6. CONCLUSION

Laboratory research confirmed both hypotheses proposed at the beginning: that Mediterranean mussels are an effective organism for biomonitoring MP fibers in the water column due to its filtration capacity and that mussels more quickly bind MP fibers into their faecal pellets compared to passive MP fiber sedimentation. As well we confirmed the presence and abundance of cigarette butts on the Croatian and Slovenian beaches, supporting the review literature that CBs represent the most common marine litter and a potential source of MP fibers in the coastal areas.

6.1 Further actions of the research

Further actions of the project withing this thesis was conducted, focused on transferring the sample collecting method to the aquaculture facilities. The installation system which was named Mussel Microplastic biomonitoring and purification System (MMS) was placed at 5 locations in Rovinj and 2 locations in Dubrovnik coast over one year period in years 2021 and 2022. The MMS included 60 mussels and a funnel with 42 cm diameter (Figure 31).

Figure 31: The MMS displayed on land (left) and in the sea (right).

6.2 Challenges and critical evaluation

Despite the meticulous work, the laboratory experiments encountered some issues. While the sampling method was relatively easy, counting microfibers in the samples proved time-consuming and challenging in accuracy. The method of visual ID under the microscope was shown to be cost-effective but very time-consuming, while human factor error potentially gets more prominent over time. Mathematical extrapolation from small samples to the entire dataset addresses the issue of human error, but it was not accurate due to the clumping tendency of MP fibers, especially in higher concentrations. In further analyses, it is sensible to find a balance between time and accuracy effectiveness by testing multiple different methods of MP counting (for example, with software for counting cells under the microscope).

6.3 Nature conservation implication and actions

In Slovenia and Croatia, beach clean-up actions are widespread but occur 2-3 times per year. Public beaches are usually under municipality concern, while natural, wild beaches are usually exposed to human activity without oversight or regular cleaning. There is no doubt that MP is present in the aquatic environment, posing a threat to the ecosystem and public health. Further actions to reduce sources of MP in the environment would be required, starting with reducing beach litter with stricter laws, law enforcement, and more thorough monitoring. However, despite monitoring, education of the masses is crucial. Human disposal habits might change if the matter concerns us directly and evokes emotions. Therefore, an excellent example of the CB amount on the beaches as marine litter is shown in Landscape Park Strunjan (Figure 32).

Figure 32: The display in Landscape Park Strunjan, showing the amount of collected cigarette butts in just 2 hours of coastal clean-up.

Another excellent example of reducing CB litter is in Žusterna Beach in Koper, where the municipality offers free use of ashtrays (Figure 33)

Figure 33**:** Žusterna Beach Koper, Slovenia, offering free use of ashtrays to reduce CBs litter.

An alternative interesting aspect of finding a solution to reduce CB litter is presented in an article by Moroz et al. (2021). Some suggestions from the article are as follows: methods for cellulose acetate recycling solutions, using smoked CB leachate solutions as a source for insecticide production for mosquito control, using smoked leachate solutions as corrosion inhibitors, as reinforcement in natural rubber, usage in the field of electric applications, as sound absorption material and other interesting ideas which would make CB reusable. Despite the interesting potential applications, it is essential to recognize the fact that the topic needs further investigation, especially in studying the long-term environmental impacts.

7. POVZETEK V SLOVENSKEM JEZIKU

Onesnaževanje s plastiko je v zadnjem desetletju pritegnilo veliko pozornosti znanstvenikov in širše javnosti. Plastični odpadki so prisotni v vseh okoljih, vključno z morjem – od priobalnih območij do globokomorskih predelov ter od vodnega stolpca do morskega dna. Izraz "plastika" se nanaša na skupino umetnih polimerov, ki so značilni po svoji lahkosti, trpežnosti in predvsem odpornosti na razgradnjo v naravi, kar omogoča njihovo široko uporabo v industriji in vsakdanjem življenju. Plastiko lahko kategoriziramo glede na njene kemične lastnosti ter velikost delcev. Slednja je pomemben dejavnik, saj ima velik vpliv na okolje. Po velikosti delcev lahko plastiko delimo na makroplastiko (delci večji od 20 mm), mesoplastiko (delci v velikostnem razponu od 5 do 20 mm), mikroplastiko (delci manjši od 5 mm) in nanoplastiko (delci manjši od 100 nm).

Ta magistrska naloga se osredotoča na vidik mikroplastike v morju. Raziskave so pokazale, da je distribucija mikroplastike v morju zelo heterogena, nanjo pa vplivajo različni okoljski pogoji in kemične lastnosti mikroplastike. Mikroplastika predstavlja potencialno mehansko in kemično grožnjo organizmom v okolju, saj ti z mikroplastiko ustvarjajo interakcije preko svojega načina življenja; mikroplastične delce lahko absorbirajo, filtrirajo, se nanje prilepijo, jih vdihnejo ali prebavijo, kar lahko povzroči notranje poškodbe in/ali toksikološki stres. Mikroplastika predstavlja tudi grožnjo prehrani, saj so fragmenti mikroplastike bili najdeni v ribolovnih in komercialnih vrstah morskih organizmov.

Kljub veliki raznolikosti plastičnih odpadkov literatura navaja, da so najpogostejši odpadek na plažah cigaretni ogorki. Podatki kažejo, da naj bi ljudje vsako leto v naravo odvrgli približno 900 milijonov ton cigaretnih ogorkov.

Glavne sestavine cigaretnih ogorkov so tobak, papir, pepel in cigaretni filter. Slednji je običajno narejen iz celuloznega acetata. Celuloza je sicer biorazgradljiva snov, vendar se v procesu acetilacije dodajo plastifikatorji, ki omejijo biodegradacijski potencial acetatne celuloze do te mere, da jo lahko uvrščamo med plastiko. Cigaretni ogorki predstavljajo kemično grožnjo, saj so v njih zaznane več kot 500 potencialno strupenih, mutagenih in kancerogenih snovi, kot so težke kovine, kadmij in arzen. Drug vidik groženj ekosistemom pa predstavljajo cigaretni ogorki kot potencialni vir mikroplastičnih vlaken, ki so v morskem okolju najpogosteje zaznani tip mikroplastike.

Glede na vsesplošno prisotnost mikroplastike se je pojavila potreba po natančnem monitoringu in metodah, ki bi učinkovito zaznale in kvantificirale mikroplastične delce v vodnih okoljih. Pregledana literatura navaja, da so učinkoviti biomonitorji prav školjke iz

rodu *Mytilus* (klapavice), ki so sicer zelo razširjen in dobro poznan organizem. Te se hranijo s filtracijo, pri čemer so zaznani njihovi odzivi na spremembe v okolju.

Glavni cilj te magistrske naloge je bil preučiti potencial filtracijske funkcije užitne klapavice (*Mytilus galloprovincialis*) pri monitoringu mikroplastičnih vlaken ter možnost uporabe te funkcije kot orodja za odstranjevanje mikroplastičnih vlaken iz vodnega stolpca v laboratorijskih pogojih, z namenom, da bi rezultate potencialno prenesli v dejansko naravno okolje.
Užitne klapavice so na vzhodni obali Severnega Jadrana zelo pogosta vrsta školjk, ki jih pogosto najdemo v naravi in ki so najbolj pogosto gojena morska vrsta v Sloveniji in na Hrvaškem. Poleg tega sta biologija in ekologija te vrste dobro poznani, kar so ključni pogoji za to, da je lahko vrsta ustrezen bioindikator. Poleg tega se klapavice dejansko odzivajo na spremembe v okolju z vedenjskimi in telesnimi spremembami.

Laboratorijski poskusi so vključevali užitne klapavice, ki smo jih pridobili iz marikulturnega objekta v Limskem fjordu na Hrvaškem. Vsem klapavicam smo za primerljivost rezultatov izmerili kondicijski indeks. Modelna mikroplastična vlakna smo pripravili iz rabljenih in nerabljenih cigaretnih ogorkov. Za vsak poskus smo pripravili suspenzijo morske vode in mikroplastičnih vlaken po enakem protokolu. Vse vzorce, ki so vključevali mikroplastična vlakna, smo vizualno pregledali in prešteli mikrovlakna pod mikroskopom pri 100-kratni povečavi. Vsak vzorec je bil preštet trikrat na 10 ml, nato pa matematično ekstrapoliran na celoten vzorec z volumnom 2 litra.

Učinkovitost filtracije klapavic smo najprej preverili s pomočjo v vodi raztopljene organske snovi. Rezultati so pokazali, da se sprememba v merjeni intenziteti svetlobe (lux) med testno in kontrolno skupino, ki ni vsebovala klapavic, hitreje spreminja v prisotnosti klapavic.

Drugi poskus je vključeval modelna mikrovlakna iz rabljenih in nerabljenih cigaretnih ogorkov, ki je potekal 15 dni. S poskusom smo želeli preveriti več vidikov, in sicer: ali obstaja razlika v hitrosti pasivne sedimentacije mikrovlaken in mikrovlaken, ki jih klapavice v procesu filtracije zlepijo v fekalni in psevdo-fekalni pelet; ali obstaja razlika med pasivno sedimentacijo in aktivno filtracijo glede na rabljenost cigaretnega ogorka ter ali je metoda zbiranja fekalnega in psevdo-fekalnega peleta s pomočjo lija dovolj učinkovita. To je bilo pomembno tudi za nadaljevanje projekta, v okviru katerega je bila magistrska naloga narejena, da bi lahko metodo zbiranja vzorcev in štetja mikrovlaken prenesli na dejansko območje marikulture na večjo skalo. Rezultati so pokazali, da klapavice hitreje nalagajo mikrovlakna v fekalni pelet v prvih 24 urah poskusa. V nadaljevanju se niso pokazale statistično pomembne razlike med hitrostjo in deležem pasivno sedimentiranih vlaken ter

aktivno filtracijo s klapavicami. To pomeni, da mikrovlakna lahko popolnoma pasivno sedimentirajo, vendar je zbiranje vzorcev in situ težje, medtem ko lahko fekalni pelet, ki ga proizvajajo klapavice, zberemo in zelo natančno pregledamo. Pri primerjavi aktivne filtracije klapavic med mikrovlakni iz rabljenih in nerabljenih cigaretnih ogorkov smo ugotovili, da klapavice hitreje in v večji meri izločajo mikrovlakna iz rabljenih cigaretnih ogorkov, kar nakazuje na njihov toksikološki odziv.

Po zaključku poskusa smo preverili tudi, koliko mikrovlaken se zadrži v notranjosti klapavic. Rezultati so pokazali, da klapavice dejansko izločijo 90 % vseh mikrovlaken skozi fekalni in psevdo-fekalni pelet, zanemarljivo majhno število vlaken pa je bilo opaziti na površini in v notranjosti drugih organov klapavic. Ostalih 10 % mikrovlaken so klapavice izločile v procesu purifikacije, ki je trajal 6 dni.

V naslednjem koraku smo preverjali hitrost purifikacije klapavic glede na čas izpostavljenosti mikrovlaknom in glede na čas purifikacije. Modelna mikrovlakna, ki smo jih uporabili v poskusu, so bila mikrovlakna iz nerabljenih cigaretnih ogorkov. Rezultati so pokazali, da klapavice največ mikrovlaken izločijo v prvih 24 urah purifikacije, nato pa delež znatno upada. Največ vlaken so izločile klapavice, ki so bile mikrovlaknom izpostavljene 4 ure, najmanj pa tiste, ki so bile začetni suspenziji z mikrovlakni izpostavljene 1 uro.

Pri pregledu natančnosti metode štetja mikrovlaken pod mikroskopom smo ugotovili, da metoda štetja manjših vzorcev in nato matematična ekstrapolacija na celoten vzorec ni najbolj natančna, saj imajo mikrovlakna tendenco zlepljanja v skupke, kar pomeni, da je možnost za napako večja. Poleg tega je pregledovanje večjih vzorcev zelo časovno potratno in zamudno, utrujenost pa lahko prispeva k večjim napakam.

Na koncu smo preverili še toksičnost rabljenih cigaretnih ogorkov s pomočjo testa kaljivosti lanenih semen. Rezultati so pokazali, da je statistično značilna razlika v kaljivosti semen med kontrolno skupino, kjer je bila prisotna le destilirana voda, in testno skupino s 3 mg CBU/l, kar pomeni, da kemijske spojine, prisotne v rabljenih cigaretnih ogorkih, dokazano pronicajo v vodo in zavirajo kaljivost semen. Nadaljnje testiranje ni bilo ključno za potrebe te raziskave, saj se tema osredotoča na druge vidike, potencialna toksičnost kemikalij v cigaretnih ogorkih pa je že dobro raziskana glede na pregledano znanstveno literaturo.

Končni sklep raziskave je potrdil obe začetni hipotezi, in sicer, da mikrovlakna v prisotnosti klapavic sedimentirajo hitreje v primerjavi s pasivno sedimentacijo, kar pomeni, da je filtracijska funkcija klapavic učinkovito orodje za odstranjevanje mikrovlaken iz vodnega stolpca. Še pomembneje podatki so pokazali, da so klapavice ustrezen organizem za

učinkovitejši in situ biomonitoring v vodnem stolpcu, še posebej na gojiščih, kjer so klapavice dejansko postavljene v vodni stolpec.

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