UNIVERZA NA PRIMORSKEM
FAKULTETA ZA MATEMATIKO, NARAVOSLOVJE IN
INFORMACIJSKE TEHNOLOGIJE

MASTER'S THESIS
(MAGISTRSKO DELO)

EFFECTS OF PMMA MICROPLASTIC ON CELLS AND TISSUE OF THE MEDITERRANEAN MUSSEL *MYTILUS GALLOPROVINCIALIS* AFTER AN EXPERIMENTAL EXPOSURE

(VPLIVI PMMA MIKROPLASTIKE NA CELICE IN TKIVA MEDITERANSKE ŠKOLJKE *MYTILUS GALLOPROVINICALIS* PO EKSPERIMENTALNI IZPOSTAVLJENOSTI)

TAMARA SOVIĆ

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Ključne besede: mikroplastika, klapavice, PMMA, indeks kondicije (IK), SOS test, hemocite

Izvleček: Vse večja razširjenost plastike predstavlja večjo obremenitev in škodo naravnemu okolju. V morskem okolju imaju mikroplastični delci (MP) bolj subtilne učinke kot večji delci, ker se njihov obseg velikosti prekriva z obsegom delcev, ki jih zaužijejo "filter feedersi" oziroma filtratorji. V tem okviru je bil raziskan vpliv velikosti dveh delcev (10 µm in 50 µm) PMMA (polimetilmetakrilata, znanega kot pleksi steklo) na ekološko pomembne klapavice *Mytilus galloprovincialis* oziroma Mediteranske klapavice. Namen te eksperimentalne študije je raziskati, ali se delci PMMA zaužijejo skozi škrge in prenesejo v hemolimfo in prebavni sistem, in na ta način vplivajo na zdravje klapavic. Zdravstveno stanje klapavic je bilo ovrednoteno z merjenjem indeksa kondicije (IK) in preizkusa preživetja klapavic po izpostavljenosti zraku (stres na stres, SOS test) po 72-urni izpostavljenosti nizke (0,1 mg/L), srednje (1,0 mg/L) in visoke (10,0 mg/L) koncentracije delcev PMMA. Učinek izpostavljenosti delcev 10 µm in 50 µm PMMA smo opazili s povečanjem skupnega števila hemocitov in zmanjšanjem vitalnosti celic z višjo koncentracijo obeh MP. Rezultati so tudi ugotovili znatno povečanje ravni vakuoliziranih hemocitov zaradi izpostavljenosti PMMA. S histološko analizo so v škrgah in prebavnih žlez izpostavljenih klapavic odkrili samo 10 µm PMMA in tako povzročili fiziološke poškodbe kot zmanjšano stanje in indeks kondicije v izpostavljeni klapavici. Ta študija dokazuje načelo, da se MP vnese v tkivo in povzroča pomembne učinke na zdravje klapavic.

Key words documentation

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Abstract: The widespread occurrence of plastic is becoming an increasing problem in the natural environment. Microplastic particles (MPs) in the marine environment have more subtle effects than bigger fragments because their size range is overlapping with that of particles ingested by filter-feeders. In this context, the effect of two-particle sizes (10 μm and 50 μm) of PMMA (polymethylmethacrylate, known as plexiglass) on ecologically significant mussel *Mytilus galloprovincialis* has been investigated. This experimental study aims to investigate if the PMMA particles are taken up into the mussel gills and transported to the haemolymph and digestive system causing the effect on mussel health. Mussel health status was evaluated by measuring the condition index (CI) and survival test of mussels in the air (Stress on stress, SOS test) after 72h exposure of low (0.1 mg/L), medium (1.0 mg/L) and high (10.0 mg/L) concentration of PMMA particles. The effect of 10 μm and 50 μm PMMA particle exposure was observed by increasing total haemocytes counts and reducing cell viability with a higher concentration of both particle sizes. The results also noted a significant increase in levels of vacuolized haemocytes as a result of PMMA exposure. Only 10 μm PMMA was detected in gills and digestive gland of exposed mussels by histological analysis, thus causing physiological damages as decreased condition and fitness index in the exposed mussel. This study provides proof of the principle that MP is taken up into tissue and causes significant effects of mussel health.

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LIST OF ABBREVIATIONS

CI - condition index
CON – control mussels
DDT – dichlorodiphenyltrichloroethane
df – the degrees of freedom
e.g. - for example
EU – European Union
F – the *F*-statistic
HIGH - mussels exposed to 10 mg/L
i.e. - in other words
LOW - mussels exposed to 0.1 mg/L
MID - mussels exposed to 1.0 mg/L
MMT - million metric tons
MPs – microplastics particles
MS – the mean sum of squares
p – the *p*-value
PAHs - polycyclic aromatic hydrocarbons
PCBs - polychlorinated biphenyls
PE – polyethylene
PET - polyethylene terephthalate
PLY - polyamide
PMMA - polymethylmethacrylate
PP – polypropylene
PS – polystyrene
PUR – polyurethane
PVC - polyvinyl chloride
SOS test – “Stress on stress” test
SS – the sum of squares
UV – ultraviolet

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1 INTRODUCTION

In today's industrial and consumer products, plastics are synthetic polymers that are used increasingly, with an estimate of an additional 335 million tonnes of world plastic production in 2016 alone (Plastics Europe, 2017). The same features that make plastic as one of the most usable materials also contribute to becoming a serious pollution threat. Plastics are the main waste that can be found in the marine environment and with an abundance of 70% in marine litter (Phillips, 2014). The large portion of plastics found in the marine environment is at the microscopic level - microplastics (MPs). Their size ranges up to 5 mm and belongs microplastics group (GESAMP, 2015).

The harmful effect of microplastics in the marine environment is based on the physical characteristics of plastics which have resulted in their ubiquitous distribution and transport by water currents and wind action. Currently, it is estimated that about 600 species, including marine mammals, seabirds, turtles, and invertebrates have been affected by plastic material in the marine environment (Gall and Thompson, 2015; Rochman et al., 2015; Wilcox, Van Sebille, et al., 2015). This is the reason why investigating the impact of microplastics on biota is essential.

In this context, this master's thesis aims to examine the influence of two-particle size (10 μm and 50 μm) of polymethylmethacrylate (PMMA) on the mussel *Mytilus galloprovincialis*. Mussel is a sessile suspension feeder, that filter about 3 liters of seawater in one hour, which is 72 liters a day, and in this way, various environmental pollutants (Famme et al., 1986) including microplastics can accumulate in its tissue. This is precisely one of the reasons why mussels are selected as a model organism in this master's thesis.

The purpose of this experimental study is to investigate whether the selected model of microplastics PMMA is uptake into the gills and digestive system and whether the ingested PMMA particles enter the cells and cause a negative effect on the mussel health. Mussel health status, especially physiological condition, and mussel fitness will be evaluated by a condition index (CI) test and a survival test of mussels in the air with the SOS test. The effect of exposure and plastic ingestion will be observed at the cellular level, haemocytes, and tissue (histological analysis) observation being done under the light microscope.

However, neither the extent of MP ingestion nor the possible pathways of transition into cells and cause effects at the cellular and tissue level are known. In this study, the effect of PMMA particles on marine organisms will be examined for the first time. This research will find out the maximum concentration and size of PMMA particles that can be released into the marine environment without any harmful effects on the mussels. Also, these studies will answer whether it is the mussels sensitive to PMMA particles, in what tissue is accumulated, and what effect it is on the mussel health.

1.1 AIMS

In this thesis, four primary aims have been set:

- to examine the influence of PMMA microplastics on mussels,
- to determine whether the selected model of PMMA microplastics are taken up into the mussel organs,
- to determine whether ingestion of PMMA microplastics can be transported from the tissues to their circulatory system,
- to examine whether ingestion of PMMA microplastics will influence feeding behavior of mussels and influence on mussels physiological condition

1.2 HYPOTHESIS

Hypothesis in this thesis are:

1. PMMA microplastics are taken up into the mussel through the gills and transported to the haemolymph and digestive system

2. PMMA particles accumulate in mussels cells and tissues, and their effect depends on the concentration and particle size applied

3. Smaller microplastic particles are causing more effect on mussel than those of larger diameters

4. Microplastic particles affect the mussels health status

5. Physiological condition and mussel fitness, after exposure to PMMA particles, is decreasing because of the impact of microplastic particles

2 MICROPLASTICS

Due to anthropogenic activity, the marine environment is polluted by large amounts of plastic debris, consisted of synthetic polymers of all shapes, colors, and sizes (Derraik, 2002; Browne et al., 2007; Moore, 2008; Barnes et al., 2009). In the oceans, plastics are dispersed by winds and currents over long distances across the globe. Under the influence of wave action, ocean temperature, and UV radiation, large plastic debris items gradually were degraded into smaller fractions, giving fragments smaller than 5 mm, generally categorized as microplastics (Browne et al., 2007; Barnes et al., 2009**;** Browne et al., 2010; Andrady, 2011). The term "microplastic" was put forward for the first time by Thompson in 2004 (Thompson et al., 2004). Microplastics are semi-synthetic plastic polymers particles, commonly used as scrubbers in cosmetics, hand cleansers, and are used in air-blasting (Thompson et al., 2004; Browne et al., 2015). There can be found two types of microplastics in the environment – primary and secondary microplastics (Sharma and Chatterjee, 2017). Their widespread occurrence and accumulation have been reported from various marine, freshwater, and terrestrial ecosystems (Andrady, 2011; Medrano et al., 2015; Erkes-Abel et al., 2017; Sharma and Chatterjee, 2017). They have also shown the potential to be ingested by different species, bioaccumulation, and further trophic chains transfer (Li et al., 2015; Kelly and Wright, 2017; Tosetto et al., 2017). Today, microplastic is ubiquitous and bioavailable in all ecosystems and represents a problem for the environment and human health. The associated environmental and health impacts have raised attention amongst scientists, the public, media, regulatory agencies, and politics (UNEP, 2016; Li et al., 2019).

2.1 PRIMARY MICROPLASTICS

Primary microplastics are the tiny particle fragments that are released as a result of the unintentional release of intermediate plastic feedstock such as pellets or nurdles. These tiny fragments occur as by-products of various processes such as maintenance of plastic or plastic-based materials, the release of dust and fibers, or when larger plastic items such as bags, toothbrushes, or bottles break down over decades (GESAMP, 2015). The plastic pellets comprise polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyethylene terephthalate (PET), polyurethane (PUR) and polyolefin particles and are lipophilic meaning they act like a sponge to other toxins in the water. Synthetic primary microplastics are used as abrasives in various industries (cosmetics, cleaning products, pharmaceuticals, and air-blasting media) (Thompson et al., 2004; Ryan et al., 2009; Sharma and Chatterjee, 2017). It can also be used in drilling liquids for oil and gas exploration, for removing rust and paint, as a drug delivery system (vector), and in dentist tooth polish (Derraik, 2002; Browne et al., 2007; Sundt et al., 2014; Lassen et al., 2015). It has been detected that many hydrophobic and aromatic compounds such as polychlorinated biphenyls

(PCBs), polycyclic aromatic hydrocarbons (PAHs) and dichlorodiphenyltrichloroethane (DDT) bond on the surface of pellets (Cauwenberghe et al., 2015). In Europe, polyethylene and polypropylene together account for about 50% of all plastic consumed. Therefore, polyethylene is likely to be reasonably abundant in the marine environment (Andrady, 2011; PlasticsEurope, 2017).

2.2 SECONDARY MICROPLASTICS

Secondary microplastics are defined as fragments of larger items that suffer fragmentation found in marine and terrestrial habitat (Thompson et al., 2004; Ryan et al., 2009). There are three essential processes that breakdown large plastics into tiny fragments. Those processes are weathering, photodegradation by ultraviolet radiation from sunlight and temperature (Arthur et al., 2009; Barnes et al., 2009; FAO, 2017). Degradation of plastic may result in leaching out of additives, designed to enhance durability and corrosion resistance (Talsness et al., 2009). In areas with reduced UV exposure and low temperature, the process of secondary microplastic generation will be slower. There are several ways how secondary microplastic can enter the environment (FAO, 2017):

- particles from textiles – enter the environment through wastewater or air when drying (Browne et al., 2011)
- soil runoff – plastics used in agricultural applications (FAO, 2017)
- fragmentation and weathering of items in landfills by UV light (FAO, 2017)
- weathering of plastic litter in coastal areas and beaches – may remain coastal sediments or transport offshore (FAO, 2017)
- abrasion of tires – generate microplastics that enter the environment through air and surface runoff (FAO, 2017)

2.2.1 Polymethyl methacrylate (PMMA)

Polymethyl methacrylate (PMMA) is a synthetic resin produced from the polymerization of methyl methacrylate. PMMA is classified as a thermoplastic; it becomes liquid at the melting point of 160°C. The advantage of thermoplastics is that they can be heated to their melting point, cooled, and reheated again without significant degradation. PMMA is often used as a substitute for glass in products such as shatterproof windows, skylights, illuminated signs, swimming pool enclosures, instrument panels, and aircraft canopies. It is known under the term plexiglass (Britannica, 2019; Creative Mechanisms, 2019).

PMMA was discovered in the early 1930s by British chemist Rowland Hill and John Crawford at Imperial Chemical Industries (ICI) in England, and it was registered under the

trademark Perspex. In Germany, 1928, Rohm and Haas Company produce safety glass under the brand Plexiglas. After discovery, new plastic took place during World War II. It was made into aircraft windows and bubble canopies for gun turrets. Today common uses include lenses, acrylic nails, paint, security barriers, medical devices, LCD screens, furniture, windows, tanks, and enclosures around exhibits. It is used so often because it is inexpensive, very scratch resistant, lighter alternative to glass, it can be cut into extremely fine shapes, but it has relatively low impact resistance and strength in general (Britannica, 2019; Creative Mechanisms, 2019).

2.3 MICROPLASTICS IN THE MARINE ENVIRONMENT

Plastics are ideal for a variety of applications because of their characteristics such as low cost, lightweight, robust, and excellent oxygen/moisture barrier properties, transparent material (Andrady, 2011). It has a wide application in every human endeavor: shipping, packaging, agriculture, automobiles, biomedical, telecommunication, building and construction, furniture, plumbing works, transportation, personal care products, aquaculture and fisheries, textile and clothing (Ismail et al., 2009; Vianello et al., 2013; Wright et al., 2013; Cozar et al., 2014; Turra et al., 2014). These different human activities can easily pollute the marine ecosystem with microplastics (Figure 1). The domestic, industrial, and coastal activities are the prime routes for the entry of the plastic litter in the marine habitat (Derraik, 2002). Pellet spills, coastal tourism, commercial fishing, and aqua industries are other ways of microplastic pollution in the marine environment (Sharma and Chatterjee, 2017). Today, the entire global fishing fleet uses plastic gear such as polyolefins (PE and PP) and nylons. About 18% of marine plastic debris found in the ocean is attributed to the fishing industry (Klust, 1982; Timmers et al., 2005; Andrady, 2011). Plastic enter water bodies via wastewater, river, or by wind currents. Terrestrial microplastic debris from land enter water bodies through extreme weather conditions like flash-flooding, the wind blows, storms, and hurricanes (Barnes et al., 2009; Cole et al., 2011). Land-based sources, including beach litter, contribute about 80% of the plastic debris (Andrady, 2011).

In 2017, the global production of plastics reached 348 million metric tons, with 64 million metric tons produced in Europe alone. China is one of the largest producers of plastics in the world, accounting for more than one-quarter of the global production (Figure 2) (Statista, 2019).

From 348 MMT of plastics that are manufactured annually, 50% of those are disposed into the environment. About 4.8 – 12.7 MMT end up in the marine ecosystem (water column, sediment, and biological tissues) through wastewater as microplastics coming from personal care products, cosmetic and pharmaceutical sources or by the degradation of larger plastic

litter (Cole et al., 2011; Andersson, 2011; Law and Thompson, 2014; Singh and Sharma, 2016; Sharma and Chatterjee, 2017; Auta et al., 2017).

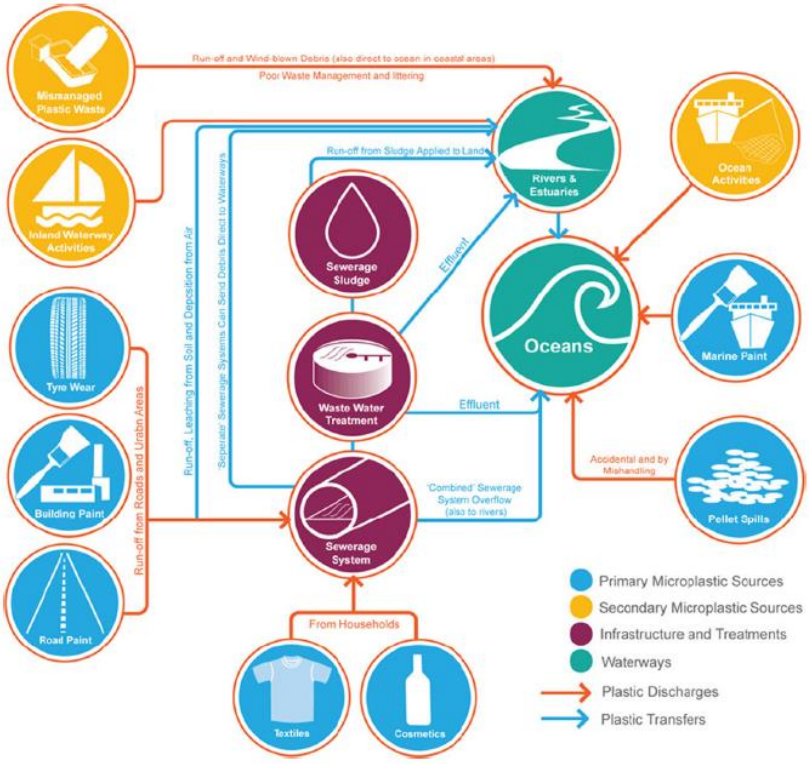

Figure 1. Major microplastic sources and pathways to the environment (Ogunola et al., 2018)

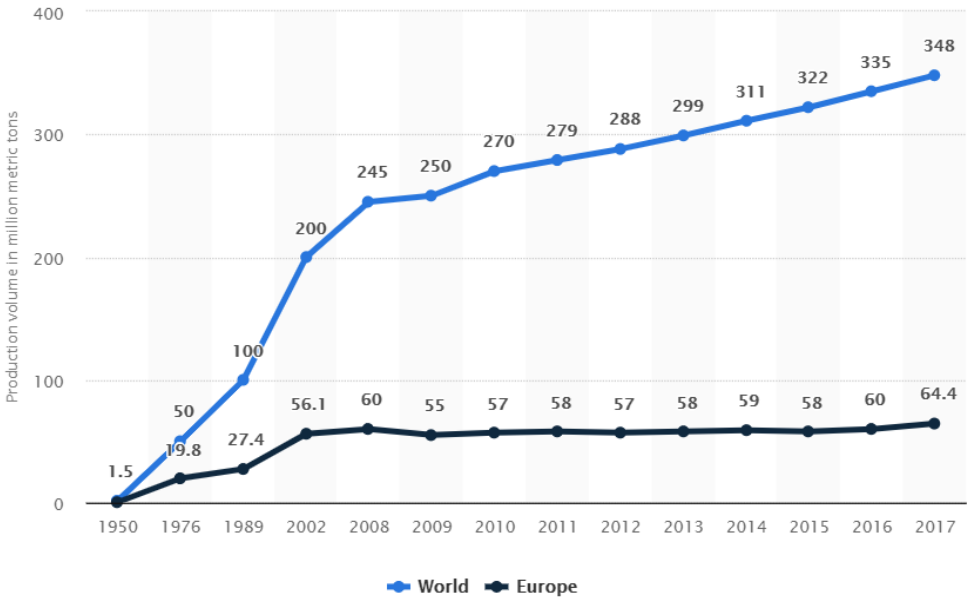

Figure 2. Production of plastics worldwide and in Europe from 1950 to 2017 in a million metric tons (MMT) (Statista, 2019)

Microplastic pollution is increasing worldwide because of the difficulty in removing it from the environment matrices due to its small size and less visibility (Auta et al., 2017).

Plastic has become ubiquitous in the marine environment and is present even in the remotest of areas. With time, plastics on the water surface accumulate in gyres, sink to the seabed and accumulate in sediments or are washed ashore and litter coastlines (Galgani et al., 2000; Moore et al., 2001; Derraik, 2002; Thompson et al., 2004; Barnes et al., 2009; Browne et al., 2010;). The geographical distribution of plastic debris is influenced by hydrodynamics, geomorphology, and human factors (Barnes et al., 2009). The first discovery of a high concentration of microplastics was in the North Pacific central gyre where the term "ocean garbage patches" was firstly originated (Kaiser, 2010; Zhang et al., 2010). This ring of marine litter is located between 135 and 155° W and 35-42° N (Moore et al., 2001).

Approximately 22.290 tons of floating plastic debris (> 33.000 particles km^{-2}) were reported to accumulate in the zone of North Pacific subtropical gyre, which includes plastic fragments, pellets, PE, PP, and thin plastic films (Law et al., 2010). In the South Pacific subtropical gyre, an estimated average of 26,898 particles km^{-2} was found (Eriksen et al., 2013a).

The distribution of plastic debris in seawater is different. Reasons for that are local wind and current conditions, coastline geography, and the points of entry into the system, such as urban areas and trade routes (Barnes et al., 2009). The plastic hotspots are generally formed in the coastal regions where industrial (shipping/maritime industries, fishery operators) and human activity (beach visitors, tourists) is high (Noren and Naustvoll, 2010; Barboza and Gimenez, 2015; Kiessling et al., 2017). The abundance of plastic debris in the Atlantic Ocean and the Mediterranean Sea was reported to be high due to both natural and human factors (Noren and Naustvoll, 2010). About 500 and 2000 items of anthropogenic debris strand on the north and South Atlantic Ocean shores per linear kilometer per year. More than six times as much plastic strands in the Mediterranean Sea and less than six times as much strand in the Southern Ocean shores (Barnes and Milner, 2005). Enclosed seas and semi-enclosed seas such as the Caribbean have high densities of plastic debris but also considerable variability. Also, high frequencies and variability can be a feature of open ocean coastlines like Brazil and Hawaii (Santos et al., 2005; Dameron et al., 2007). One of the critical reasons for interannual variability in abundance is connected to changes in oceanic circulation driven by El Niño events (Matsumura and Nasu, 1997; Morishige et al., 2007).

Low-density microplastics (polypropylene and polyethylene) are likely to be found in the sea-surface microlayer (Gregory, 1996; Derraik, 2002). Plastics can sink to the seabed under the weight of fouling by a wide variety of bacteria, algae, animals, and accumulated sediment. Plastics have been found on the seabed of all seas and oceans across the planet, but macro-debris is still very rare in the Southern Ocean, particularly in deep water (Gregory

et al., 1984; Barnes et al., 2009). In contrast to floating debris, which accumulates in frontal areas, bottom debris tends to become trapped in areas of low circulation and high sediment accumulation. Waste that reaches the seabed may have already been transported a considerable amount of distance, only sinking when weighed down by fouling (Barnes et al., 2009). High-density microplastics (e.g., polyvinylchloride, polyester, polyamide) are likely found in large quantities in the benthos (Barnes et al., 2009). Claessens et al. (2011) take off the sediment samples from the Belgian coast. The highest microplastic (<1 mm in diameter) concentration (~ 391 microplastics/kg of dry sediment) was found in a harbor sediment sample, probably due to the local anthropogenic activity, river run-off and trapping of sediments. Deep-sea sediments collected from the Atlantic Ocean, the Mediterranean Sea, and the Indian Ocean have been found to contain microplastics in the form of fibers, and abundance was up to four orders of magnitude than in contaminated sea-surface water (Van Cauwenberghe et al., 2013; Pham et al., 2014; Tubau et al., 2015). Mediterranean sites tend to show the highest densities due to densely populated coastlines and shipping in coastal water. Because of limited tidal flow or water circulation, a lack of dispersion of plastics is present (Barnes et al., 2009). Due to turbulence, microplastics on the seabed may resuspend and enter the water column again (Cole et al., 2011).

Plastic can directly enter the marine environment through coastal tourism, commercial and recreational fishing, and maritime industries (e.g., aquaculture) (Figure 3). Tourism and recreational activities represent the primary source of plastic litter being discarded along beaches and coastal resorts (Derraik, 2002).

Marine plastic debris may cause a reduction in income. Due to marine litter, Hawaii and Maldives are facing declines in tourist numbers and associated revenues (Thevenon et al., 2014). However, marine debris found on beaches doesn't always have to be directly connected to human activity. Ocean currents can also bring marine debris to the shores. This debris on beaches will degrade rapidly to microplastic due to high oxygen availability and direct sunlight exposure (Moore, 2008; Barnes et al., 2009; Andrady, 2011). Fishing gear, lost or discarded, is the most common plastic debris in the marine environment. Today, fishing gear is made of plastic monofilament and nylon netting and is neutrally buoyant and can drift at variable depths within oceans. It may cause entanglement of marine biota known as „ghost fishing“ (Lozano and Mouat, 2009).

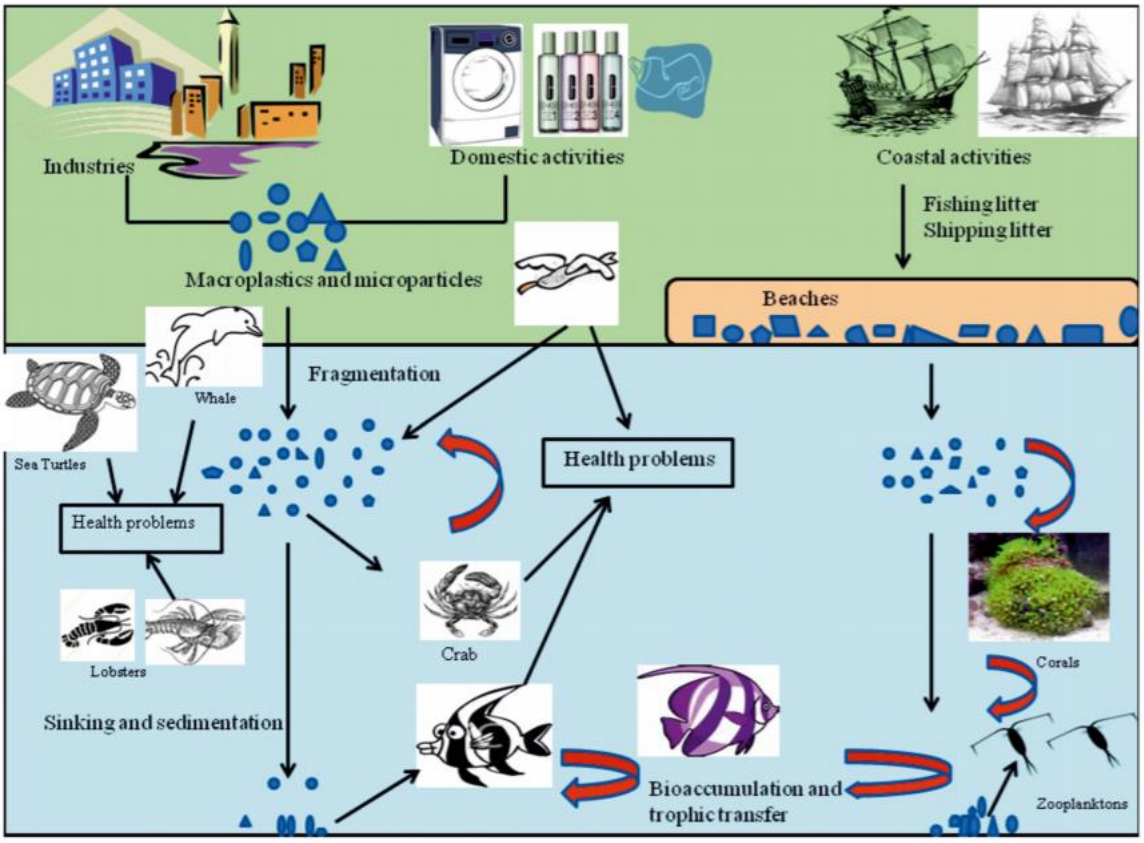

Figure 3. Environmental fate of microplastic (Sharma and Chatterjee, 2017)

Macro- and microplastic can also be found within beach sediment. The presence of a high concentration of plastics on a shoreline can dramatically alter the physio-chemical properties of the beach sediment (Carson at el., 2011). It increases the permeability of the sediment, decreases its heat absorbance so the sediment would reach lower maximal temperatures than sediment without plastics. Lower temperatures might affect sex-determination in turtle eggs, and greater permeability will increase the probability of desiccation in sediment-dwelling organisms (Cole et al. 2011).

The leading cause of variations in the density of different plastic debris in the marine environment is the formation of microbial biofilm on the polymer surface, which leads to colonization of polymer (by algae or invertebrates such as crabs, lobsters, sea urchins, worms starfish, and jellyfish) and ultimately increases the density of plastic debris (Andrady, 2011). Plastic waste can be vectors for microbial pathogens or chemical pollutants such as synthetic additives (i.e., bisphenol A), heavy metals, or persistent organic pollutants**.** It can also transport non-indigenous species to new locations and distribute algae associated with red tides (Mato et al., 2001; Barnes, 2002; Maso et al., 2003). Persistent organic pollutants occur in seawater at deficient concentrations. By process of partitioning, organic pollutants are picked up by microplastics, and because of their hydrophobicity, their concentration in the microplastic litter is several times higher than in seawater. These contaminated plastics,

when ingested by marine species, present a way by which the persistent organic pollutants can enter the marine food web (Andrady, 2011). Microorganisms may colonize the microplastic surface, constituting the "plastisphere" (Zettler et al., 2013). Those colonized particles in a biofilm can favor the spreading of microbes or bacterial pathogens through the sea (Andrady, 2011; Reisser et al., 2014; Galgani et al., 2014; Kirstein et al., 2016). The potential of spreading organisms through the sea on microplastic particles is of significance because the prevailing currents that travel from northern Australia and south Indonesia during summer and from Somalia, India and North Indonesia during winter could potentially transport an extensive range of species to less biodiverse, mid-ocean islands (Barnes et al., 2009). Also, pollution by microplastics can affect ecological processes such as primary production, biogeochemical cycling, or bioremediation if those microorganisms are involved. They influence the metabolic pathways and enzymes that drive such processes (Caruso et al., 2018).

Consequences on marine life have been reported for several years: accumulation of plastic debris, ingestion, entanglement from zooplankton to mammals (Laist, 1987; Moore, 2008; Cole et al., 2011). Microplastics can be transferred through the food web to higher trophic levels (Thompson et al., 2004; Browne et al., 2008; Cole et al., 2011; Wright et al., 2013). Microplastics are in the same range as plankton and because of that can be easily ingested by invertebrates (polychaetes, crustaceans, echinoderms, bryozoans, bivalves) and by vertebrates (fish and sea birds) (Derraik, 2002; Thompson et al., 2004; Ward and Shumway, 2004**;** Browne et al. 2007; Moore, 2008). MPs have also been detected in planktonic organisms such as zooplankton, Chaetognatha, larval fish, copepods, and salps (Bern, 1990; Moore et al., 2001; Fendall and Sewell, 2009). Lower-trophic level organisms are susceptible to ingesting microplastics, as many of them are indiscriminate feeders with limited ability to differentiate between plastic particles and food (Moore, 2008). As low-density microplastics are buoyant and abundant near the sea-surface, they will be widely available to planktonic organisms, including the larval stages that reside within the euphotic zone (Gregory, 1996; Fendall and Sewell, 2009). Many studies have been done to prove an impact on marine organisms. First plastic fragments were identified in the guts of sea birds in the 1960s (Ryan et al., 2009; Thompson et al., 2009b). Organisms at the higher trophic levels (marine mammals) have been found to ingest microplastics transported by prey items (Eriksson and Burton, 2003). Microplastic particles were recorded in the guts of scat of fur seals and Hooker's sea lions (McMahon et al., 1999). Microplastic entanglement impacts marine mammals, reptiles, birds, and fish. Between 57,000 and 135,000 pinnipeds and baleen whales globally are entangled annually. In many cases, it leads to acute and chronic injury or death **(**Butterworth et al., 2012; Allen et al., 2012; Nelms et al., 2015).

Human health can also be impacted by microplastics. By using everyday products that contain microplastics such as cosmetics, toothpaste, scrubs, hand washes, human health is

affected. German Federal Institute for Risk Assessment evaluated products that contain PE microplastic particles. They concluded that prolonged use of products, such as peelings and shower products that contains microplastic particles larger than 1 μm, lead to the absorption of PE and PP particles in tissues, which ultimately results in skin damage (BfR, 2015). Consuming commercial marine organisms such as mussels, oysters, fish, crabs, cucumbers, or products, such as sea salt, can have consequences for the health of consumers (Sharma and Chatterjee, 2017; Phuong et al., 2018). That is why monitoring the presence of MPs in marine organisms is of high importance (Phuong et al., 2018; Seth and Shriwastav, 2018). Seth and Shriwastav 2018, analyzed eight brands of the commercial sea salt. The microplastic particles were found in all eight brands, and concentration ranged from 103 ± 39 to 56 ± 49 particles kg^{-1} of salt. It is expected that contaminated seawater was the primary source for the presence of particles in sea salts. Concentrations of these particles from Indian salts were lower than from China but similar to Spanish and Turkish salts (Seth and Shriwastav, 2018).

2.3.1 Microplastic in the northern Adriatic Sea

The northern Adriatic Sea is the shallowest part of the Adriatic continental shelf, with maximum depths of less than 70 m and a mean depth of 33.5 m (Franco and Michelato, 1992; Vianello et al., 2018). Due to the relatively small volume (635 km^3), the northern Adriatic is under the strong influence of external factors affecting seasonal and long-term fluctuations in that aquarium. These are temperature fluctuations, annual rainfall, rivers flow, winds, evaporation, flow, and water column collapse (Cushman-Roisin et al., 2001; Supić et al., 2012).

The northern Adriatic Sea receives significant freshwater input from several rivers along the northeastern coast of Italy, the most important being the Po river (Supić and Ivančić, 2002; Vianello et al., 2018). In the first place, it delivers enormous quantities of nutrients that can cause eutrophication, resulting in hypoxia, less anoxia. In addition to nutrients, the river also brings large amounts of pesticides and wastes from the most industrialized part of northern Italy. Significant local influence on the quality of the water and pollution has large ports such as Venice, Trieste, Koper, Pula, and Rijeka; refineries and large industries (Rijeka and Venice Lagoon). Also, pollution of heavy metals like mercury, along the Italian coast, affects the quality of the sea. The northern Adriatic Sea is also subject to heavy marine traffic from merchant ships, supplier vessels for offshore activities (e.g., gas extraction), ferries, fishing vessels and recreational craft (Magaletti et al., 2018; Vianello et al., 2018).

Vianello et al. (2018) research Northwestern Adriatic in 2014 (the lagoon of Venice and the Po River). Microplastics were found in all samples (floating microplastics in seawater), most common polymers were polyethylene and polypropylene, and most of the particles were secondary microplastics (83,5%). The highest concentration observed was 10.4 particles

m^{-2}. Zeri et al. (2018) observed macro- and microplastics abundances in the Adriatic Sea (including the Gulf of Venice and the Gulf of Trieste (Slovenian waters). Average abundance of microplastics were 315,009 ± 568,578 items km^{-2} (217 ± 575 g km^{-2}). Microplastics abundance was significantly higher in nearshore (≤ 4 km) than in offshore waters (> 4 km).

The abundance of microplastic in the Adriatic Sea counted by Suaria et al. (2016) ranges from 0.4 ± 0.7 to 1.0 ± 1.8 items/m^3 with polyethylene being reported as the predominant polymer with an overall relative abundance of 52% of the total amount of detected polymers, followed by PP (16%) and synthetic paints (7%). The presence of paint and paraffin wax suggests high ship-based pollution in the Adriatic Sea.

Gomiero et al. (2019) assessed the levels and polymeric composition of microplastics in the native population of mussels *Mytilus galloprovincialis* collected in coastal and in offshore areas in the northern Adriatic Sea. The most recurring polymer type was PE followed by PP, PET, and equal amounts of PS, PLY, and PVC. The highest microplastic particle accumulation was observed in organisms collected along with coastal sites (1.06–1.33 items/g WW) compared to the offshore areas (0.65–0.66 items/g WW).

In the assessment of floating litter in the Croatian part of the middle Adriatic Sea by Palatinus et al. (2019), the average concentration of floating micro litter was 127 thousand particles/km^2. 14 different types of synthetic polymers were identified where most of them were made of PE (66.6%) and PP (19.7%). Fragments of synthetic rubbers (EPDM and EPR), polyacrylates (PMMA), polystyrene (PS), polyvinyl chloride (PVC), and polyethylene terephthalate (PET) were all accounted for less than 1% (1-3 particles in total).

Comparing to the middle Adriatic, in the result from the DeFishGear Project by Palatinus et al. (2017), the highest result of floating litter was in northern Adriatic ($795x10^3$ particles km^2).

2.4 REDUCING PLASTIC

Marine litter is a rapidly growing concern at sea and shores alike, having a tremendous impact on the environment. It is defined as any solid material which has been discarded or unintentionally lost on beaches, on coastlines, or at sea. It comprises a wide range of materials including plastic, metal, wood, glass, paper, and rubber. Marine litter is not only an aesthetic problem but incurs socioeconomic costs, threatens human health and safety, and has impacts on aquatic organisms. Governments need to improve waste management practices, develop new policies for a circular model of design and production of plastics, raise awareness among consumers. Several international conventions and agreements have been introduced to prevent or control the release of marine litter, including plastics and

microplastics, into the marine environment. Some of them are the OSPAR convention and the HELCOM (Helsinki Convention) (OSPAR, 2019; HELCOM, 2019).

OSPAR is the mechanism by which 15 Governments and the EU cooperate to protect the marine environment of the North-East Atlantic. The OSPAR convention comes to force on 22 September 1992 by binding Oslo and Paris Convention. The agreement was signed and ratified by all of the contracting parties to the original Oslo or Paris Conventions. The fifteen Governments are Belgium, Denmark, Finland, France, Germany, Iceland, Ireland, Luxembourg, The Netherlands, Norway, Portugal, Spain, Sweden, Switzerland, and the United Kingdom. There are six working areas of OSPAR: 1. Biological diversity and ecosystems, 2. Hazardous substances and eutrophication, 3. Human activities, 4. Offshore industries, 5. Radioactive substances, 6. Cross-cutting issues.

In 2014 OSPAR agreed to develop a Regional Action Plan (RAP) for Marine Litter along with an implementation plan to achieve its objective to reduce amounts of marine litter significantly. The RAP focuses on both sea-based and land-based sources of litter, as well as considering removal actions and education and outreach. It will be implemented over the period 2014-2021. The RAP contains 55 collective and national activities that aim to address both land-based and sea-based sources, as well as education and outreach and removal actions. The critical action areas include Port Reception Facilities, Waste from fishing industry, Fines for littering at sea, Fishing for litter, Abandoned and lost fishing gear, Floating litter hotspots, Education and outreach, Improved waste management, Sewage/stormwater run-off, Reduction of single-use items, Removal of microplastics from products/zero pellet loss, Redesign of harmful products (OSPAR, 2019).

HELCOM (Baltic Marine Environment Protection Commission - Helsinki Commission) is the governing body of the Convention on the Protection of the Marine Environment of the Baltic Sea Area, known as the Helsinki Convention. The Convention covers the whole of the Baltic Sea area, including inland waters as well as the water of the sea itself and the seabed. The Helsinki Convention was adopted in 1992 by Denmark, Estonia, the European Union, Finland, Germany, Latvia, Lithuania, Poland, Russia, and Sweden. There are ten action areas of HELCOM: 1. Agriculture, 2. Fisheries, 3. Industrial and municipal releases, 4. Marine litter and noise, 5. Marine protected areas, 6. Maritime spatial planning, 7. Monitoring and assessment, 8. Response to spills, 9. Species and biotopes, 10. Shipping (HELCOM, 2019).

In 2013 HELCOM developed a regional action plan on marine litter, adopted in 2015, to achieve a significant reduction of marine litter by 2025 and to prevent harm to the coastal and marine environment. The regional action plan for marine litter should enable concrete measures for prevention and reduction of marine litter from its primary sources; develop joint indicators and associated targets related to quantities, composition, sources, and

pathways of marine litter; and to identify the socio-economic and biological impacts of marine litter (HELCOM, 2019).

2.5 MEDITERRANEAN MUSSEL Mytilus galloprovincialis

2.5.1 Biological characteristics of the mussel *Mytilus galloprovincialis*

The species *Mytilus galloprovincialis* is taxonomically classified as (WoRMS, 2019):

Phylum: *Mollusca*

Class: *Bivalvia*

Order: *Mytiloid*

Family: *Mytilidae*

Genus: *Mytilus*

Species: *Mytilus galloprovincialis*

Mytilus galloprovincialis is the dominant mussel of the genus *Mytilus* along the eastern Adriatic coast (Pavičić-Hamer et al., 2016). It is an indigenous species of the Adriatic Sea that most inhabit coastal areas and tidal zones. *Mytilus galloprovincialis* has a shell of black or dark blue color that can often be covered by various fouling. The shells are uniform and connected by an adductor. It feeds by filtering seawater and removing organic matter from it. The nutrient particles are trapped on the gills and then ingested. Each shellfish filters up to 6 liters of water over one hour (Yildiz et al, 2006; Peharda et al., 2007; Marušić et al., 2009,). Shellfish can grow up to 15 cm in length. The sex of the individuals can be determined by the color of the gonads contained within the shell. Males have gonads of milky white color, while females have gonads of orange-red color (Bayne, 1976).

The Mediterranean mussel is also indigenous to the Mediterranean, the Black Sea and the shores of the Eastern Atlantic. It has also been found along the coasts of North America, Japan, Australia, Chile, and South Africa, where it has been introduced for anthropogenic activities and is also considered an invasive species. Figure 5. represent the distribution map of *Mytilus galloprovincialis.* The green area represents a native range of species, the red area represents the confirmed invaded regions and the yellow area represents the possible native range.

Figure 4. *Mytilus galloprovincialis* (Paiva, 2014)

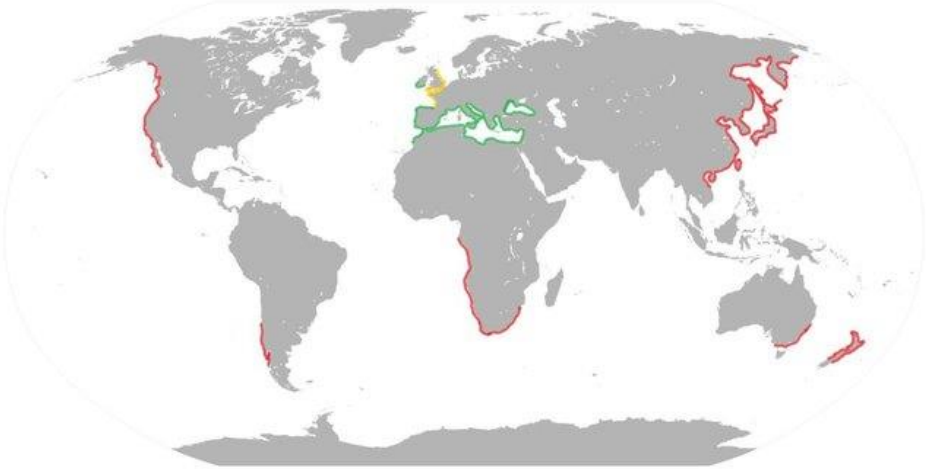

Figure 5. Distribution map of *Mytilus galloprovincialis* (Paiva, 2014)

M. galloprovincialis is widely distributed in urbanized coastal environments thus likely being exposed to MP sources for its entire life cycle; it is also a species of high ecological and commercial importance (Viarengo et al., 2007).

2.5.2 Mussel as a model organism

Mussels have been used as biological indicators in the monitoring of anthropogenic pollution trends in coastal waters. *Mytilus galloprovincialis* is used as a sentinel organism in several biomonitoring programs under UNEP in the Mediterranean Sea, OSPAR at the North and Baltic Sea, and U.S. Mussel Watch Project (Beyer et al., 2017; Marbef, 2019). Field investigation of microplastics in mussels is currently spread over 16 countries, especially in European countries (Germany, France, Belgium, the Netherland, Italy..) (Li et al., 2019).

Mussels have characteristics of ideal bioindicators. They are globally distributed, easily accessible, and have a high tolerance to a wide range of environmental parameters, including salinity, temperature, oxygen levels, and food availability (Bayne, 1976; Li et al., 2019). Mussels form beds in shallow waters from whey they easily can be collected, and they are sessile filter feeders that reflect location-specific information (von Moos et al., 2012; Brate et al., 2018; Phuong et al., 2018). Because of sessile characteristics, they have instant exposure to pollutants (pesticides, toxins, metal(loid)s, hydrocarbons) that can have several biological impacts on their physiology (Livingstone and Pipe, 1992; Höher et al., 2015). The mussel filters about 3 liters of seawater in one hour, which is 72 liters a day, and in this way, various environmental pollutants can accumulate in their tissue (Turk, 2011). Mussels can efficiently accumulate chemical pollutants from seawater to provide an integrative measure of the concentration and bioavailability of seawater pollutants *in situ* (Beyer et al., 2017). Qu et al. (2018) observed the consistency of the proportion of morphotype and polymer types in mussels and seawater. Results suggest that the microplastics in mussels can reflect the real pollution status in the environment in terms of morphotype and polymer types. Furthermore, the abundance of microplastics (microbeads) in mussels was significantly higher in the high concentration exposure group than the low concentration group. Microplastic abundance in mussels is closely related to human activity, and mussels from areas with intensive human activities contain significantly higher numbers of MP than mussels in mariculture areas (Li et al., 2016). A higher number of microplastics in mussels indicates that microplastic pollution in mussels is closely correlated with the degree of pollution in coastal habitats and can reflect the real abundance of microplastics in the environment within a certain size range (Li et al., 2019).

Browne et al. (2008) investigate ingestion, translocation, and accumulation of microplastic in the mussel *Mytilus edulis.* Within 12h, mussels exposed to microplastic particles had accumulated polystyrene microspheres in their gut cavity and digestive tubules. After 3 days, ingested microplastic accumulated in the circulatory fluid of mussels and particles were found in the haemolymph and haemocytes.

Von Moos et al. (2012) observed the presence of high-density polyethylene (HDPE) in the blue mussel *M. edulis*. They observed the presence of HDPE particles on gills and inside the digestive system. HDPE particles were seen on gills, indicating that they were trapped from the water column. Particles were also found in the intestine, in the lumina of the digestive gland and in endocytotic vacuoles of digestive cells which suggests that particles were taken up via mouth, transported to the gastrointestinal tract and internalized into cells of the digestive system by endocytosis.

2.5.3 Haemocytes

Mollusc haemocytes are involved in cellular and humoral defense reactions. Foreign material can be phagocytosed or encapsulated by haemocytes depending on its size (Carballal et al., 1997). Phagocytosis is one of the most essential and effective cellular defense mechanisms against pathogen invaders and foreign materials in bivalve (Carballal et al., 1997; Ordas et al., 2000). Hydrolytic enzymes degrade phagocytosed materials. In some bivalves, phagocytosis is associated with the production of reactive oxygen intermediates such as superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl radical (Carballal et al., 1997).

By morphological criteria, two basic cell types of bivalve haemocytes are distinguished – hyalinocytes and granulocytes (Cheng, 1981; Carballal et al., 1997; Pila et al., 2016). Haemocytes constitute the cellular component of the haemolymph, but they are also resident in other sites such as the connective and vascular tissues (Cheng, 1981; Loker, 2010). Haemocytes have essential roles in phagocytosis, transport of nutrients and internal defense reactions, encapsulation, production of cytotoxic molecules, in vital processes such as nerve repair, wound healing, and shell repair (Loker et al., 1982; Franchini and Ottaviani, 2000; Mount et al., 2004; Hermann et al., 2005; Lacchini et al., 2006; Humphries and Yoshino, 2008; Hanington et al., 2010). The number of circulating haemocytes may be depleted in these roles and must be replenished by hematopoiesis (Pila et al., 2016; Andreyeva et al., 2019).

The haemolymph of *Mytilus galloprovincialis* contains both agranulocytes (hyalinocytes) and granulocytes (Carballal et al., 1997; Andreyeva et al., 2019). Agranulocytes (hyalinocytes) in mussels possessed round shape, a large basophilic nucleus with a rough structure, and a narrow cytoplasm surrounding it. They are less active in the cellular immune response, such as phagocytosis. Granulocytes are relatively large, ameboid shape with small eccentric nuclei containing heterochromatin, the cytoplasm contains granules (Andreyeva et al., 2019). In *M. galloprovincialis,* both granulocytes and hyalinocytes contributed to spontaneous ROS production, but granulocytes seem to be the most active cells. ROS production has been directly associated with phagocytosis (Andreyeva et al., 2019).

The most used method, to verify the cytotoxic effect on haemocytes, is cell viability. Cell viability is the quantification of the number of live cells and is usually expressed as a percentage of the control (King, 2000). Assay to measure cell viability is used to monitor the response and health of cells in the organisms after treatment with various stimuli.

3 MATERIALS AND METHODS

3.1 SAMPLING SITE AND THE MODEL ORGANISM

The effect of microplastic was studied in mussels collected at the mariculture area of Lim Bay located in Istria (the northern Adriatic Sea, Croatia). Lim Bay is a 10-km-long estuary of the river Pazinčica, which plunges into the Lim, forming a channel-like bay (Pavičić-Hamer et al., 2016).

Mussels were sampled on May 3, 2019, at the mariculture station in the Lim Bay (45°07′50″N 13°44′10″E). During the weekend, mussels were acclimated in the pools with circulating seawater in the Center for Marine Research, Ruđer Bošković Institute in Rovinj. In May, the seawater temperature was 15°C and salinity 36.3.

3.2 EXPERIMENTAL SETUP AND SAMPLING PROCEDURE

The experiment was set in 7 pools – 1 control pool, three pools for PMMA particles size 10 μm, and three pools for 50 μm PMMA. Polymethylmethacrylate, with nominal diameters of 10 and 50 mm (PMMA) were obtained from Microbeads SA, Norway. Each pool was contained of 50 mussels (N=350) in the total volume of 37 L. Samples of mussels are exposed to different concentrations (0.1, 1, and 10 mg/L) of PMMA microplastic particles for three days. Seawater was changed in pools every 24h to reduce extra stress, and pools were aerated. The concentration of PMMA particles was added to seawater every day. Every day, from each pool, three mussels were taken to determine the total number of haemocytes. After three days of exposure to the microplastic particles, 30 mussels were chosen for the SOS test, five mussels for condition index, and six mussels for histological analysis.

3.3 CONDITION INDEX

For calculating the condition index (CI), five mussel samples were taken from each pool. CI was determined according to Hickman and Illingworth (1980). The length, width, and height of each mussel were measured. The mass of closed shells, the mass of shells without water, and the weighed mass of wet tissue and shellfish were excavated. CI is calculated according to the formula:

CI (%): wet meat weight (g) × 100 / whole mussel weight (g)

3.4 SOS TEST

For the SOS test, 30 samples of mussels were taken from each pool. The SOS test represents the mussel's survival to air exposure without water. The test is set in a way that we place filter papers onto the tray together with mussels on top of them and then putting them in a wet laboratory to track the number of surviving mussels every day. Dead mussels were observed by observing of opened shells.

3.5 HAEMOLYMPH ANALYSES

The valves were slightly opened with scissors and held open with a pipette tip, excess water was drained. Haemolymph (0.1-0.5 mL) from the anterior adductor muscle was withdrawn with a 25-gauge needle and a 1-mL plastic syringe at 24, 48, and 72h of the experiment and immediately transferred into individual plastic tubes. All samples were kept on ice to prevent haemocyte clumping.

3.5.1 Total haemocyte count (THC)

Total haemocyte count (THC) was performed on aliquots of haemolymph (50 mL) fixed with Baker's formaldehyde (1:1, 4%) by counting cells in an improved Neubauer chamber. Microscope Nikon Microphot – SA was used for counting the haemocytes.

3.5.2 Cell viability

To verify if microplastics exerted a cytotoxic effect on haemocytes, cell viability of haemocytes from control and treated mussels was assessed. Aliquots of unfixed haemocytes (20 µL) were stained with trypan blue and it was found that unstained cells represented the viable cells in the suspension (Torre et al., 2013b; Pagano et al., 2017). Microscope Nikon Microphot – SA was used when counting the haemocytes.

Cell viability was calculated using the formula:

Viable haemocytes (%) = Number of viable haemocytes x 100/Total haemocytes number

3.5.3 Multinucleated haemocytes

The occurrence of vacuolation as well as ‘multi nuclei’ (multinucleated haemocytes, micronuclei, and binucleated cells) and deformation of the nucleus such as blebbing (Fenech et al., 2003; Hoher et al. 2015) were assessed (Fig. 14b). Microscope Nikon Microphot – SA was used when counting the haemocytes.

3.6 HISTOLOGICAL ANALYSIS

For histological tissue analysis, digestive glands and gills have been cut off with scissors. The tissues were suddenly frozen in N-hexane, pre-chilled in liquid nitrogen. So processed samples were stored at -80 ºC until the preparation of histological preparations. Before cryosection, the samples were attached to the microtome carrier and blended into medium O.C.T. TM (Microm Inc. GmbH, Germany) (Kovačić, 2018). Tissue samples were placed on a cryotomy carrier (Zeiss Hyrax C 50, Microm GmbH, Germany) previously cooled to -30 °C. For each tissue sample, 10 μm thick-cut preparations were prepared. The frozen cross-digestion of digestive glands was placed on the exposed slides heated to room temperature. The preparations were washed with hematoxylin and eosin (Sigma-Aldrich, USA) solution at room temperature. After staining, the preparations were incorporated into glycerol gelatin (Sigma - Aldrich, USA) and observed under a polarization microscope. The occurrence and localization of microplastics were assessed through polarized light microscopy (Zeiss Axio Scope, 100×, and 400× magnification).

3.7 STATISTICAL ANALYSIS

Statistical analysis of different parameters – total haemocyte count, cell viability, vacuolized cells, condition indices, was analyzed with ONE WAY ANOVA (Statistica 9.0).

A post hoc Tukey HSD test (Statistica 9.0) was used to determine the significant difference with $p \leq 0{,}05$ between groups of different concentrations (CON, LOW, MID, HIGH).

4 RESULTS

4.1 CONDITION INDEX

Figure 6. shows condition indices of control mussels (CON) and mussels exposed to 0.1mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 10 µm PMMA particles. The median value of condition index (CI) of control mussels (CON) is higher when compared to median values of condition indices of mussels exposed to 0.1mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of microplastics 10 µm PMMA. The median value of CI of mussels exposed to 10 mg/L (HIGH) of 10 µm PMMA is the lowest than condition indices of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW) and 1.0 mg/L (MID) concentration of microplastics 10 µm PMMA.

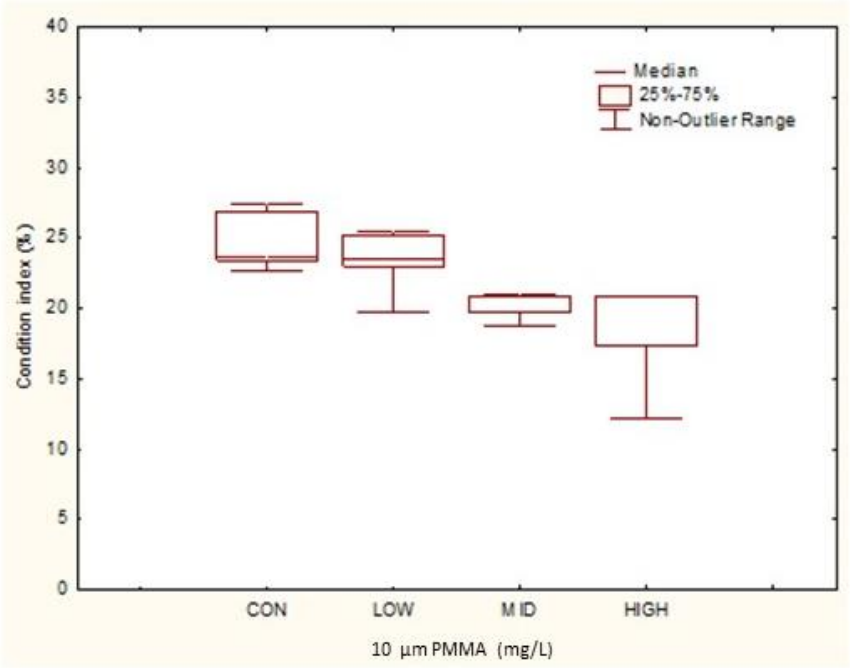

Figure 6. Condition index (%) of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 10 µm PMMA particles

Considering to control mussels (CON), CI of mussels under the influence of 10 µm PMMA is lower, which is proved with statistical significance. One-way ANOVA test (Statistica 9.0) (Table 1) shown statistical differences between different groups of mussels under the influence of 10 µm PMMA (one-way ANOVA, F= 6.785, p = 0.003). The influence of 10 µm PMMA has a bigger impact on mussel condition indices (SS= 128.405), than 50 µm PMMA (SS= 106.506) (Table 1). Considering to control mussels (CON), CI of mussels under the influence of 50 µm PMMA is lower, which is proved with statistically significant. One-way ANOVA test (Statistica 9.0) shown statistical differences between different groups of mussels under the influence of 50 µm PMMA (one-way ANOVA, F= 15.837 p = 0.0001).

Table 1. Statistical analyses (ONE WAY ANOVA, Statistica 9.0) of mussel condition indices. Significants are bolded.

Effect	SS	df	MS	F	p
PMMA 10	128.405	3	42.802	6.785	**0.003**
PMMA 50	106.506	3	35.502	15.837	**0.0001**

* SS – the sum of squares; df – the degrees of freedom; MS – the mean sum of squares; F – the *F*-statistic; p – the *p*-value

Statistically significant differences between groups are calculated with post hoc Tukey HSD, Statistica 9.0 (Table 2). Statistically significant differences are shown between CON and MID (p= 0.038), CON and HIGH (p= 0.005) and LOW and HIGH (p= 0.031).

Table 2. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of mussel condition indices exposed to 10 µm PMMA particles. Significants are bolded.

The concentration of 10 µm PMMA	LOW	MID	HIGH
CON	0.811	**0.038**	**0.005**
LOW		0.192	**0.031**
MID			0.754

Figure 7. shows CI of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID), and 10 mg/L (HIGH) concentration of microplastics 50 µm PMMA. The median value of CI of control mussels (CON) is higher when compared to median values of CI of mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID), and 10 mg/L (HIGH) concentration of 50 µm PMMA. The median value of CI of mussels exposed to 0.1 mg/L of 50 µm PMMA is the lowest than CI of control mussels (CON) and mussels exposed to 1.0 mg/L (LOW) and 10 mg/L (MID) concentration of 50 µm PMMA particles (Figure 7).

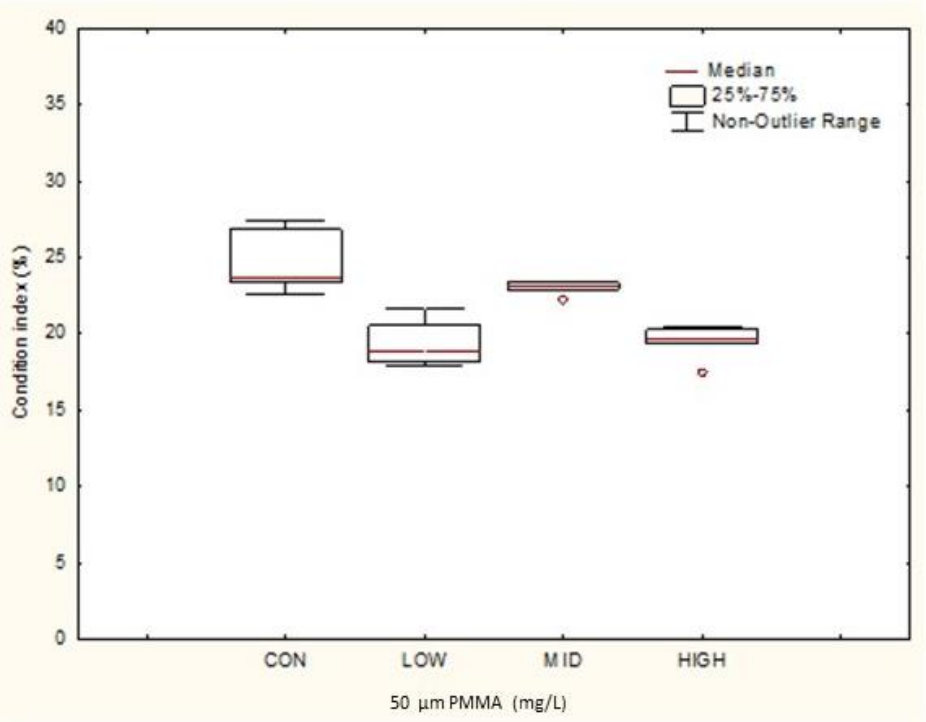

Figure 7. Condition index (%) of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 50 µm PMMA particles

Statistically significant differences within different groups are calculated with post hoch Tukey HSD, Statistica 9.0 (Table 3). Statistically significant differences are shown between CON and LOW (p= 0.0003), CON and HIGH (p= 0.0003), LOW and MID (p= 0.008) and MID and HIGH (p= 0.009).

Table 3. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of mussel condition indices exposed to 50 µm PMMA particles. Significants are bolded.

The concentration of 50 µm PMMA	LOW	MID	HIGH
CON	**0.0003**	0.268	**0.0003**
LOW		**0.008**	0.999
MID			**0.009**

4.2 SOS TEST

Figure 8. and Figure 9. represent cumulative survival and cumulative mortality of control mussels and mussels exposed to different concentrations of microplastic 10 µm and 50 µm PMMA particles through days. The lowest survival and the most significant mortality have mussels exposed to a concentration of 10 mg/L of 10 µm PMMA. After ten days, all the mussels were dead. The most significant survival and the lowest mortality have mussels in the control group. Cumulative survival was 20 days. Mussels exposed to a concentration of 0.1mg/L and 1.0 mg/L of 10 µm PMMA have survived for 15 days. Mussels exposed to concentrations of 0.1mg/L and 10 mg/L of 50 µm PMMA had survived for 14 days. Mussels exposed to a concentration of 1.0 mg/L of 50 µm PMMA had survived for 12 days.

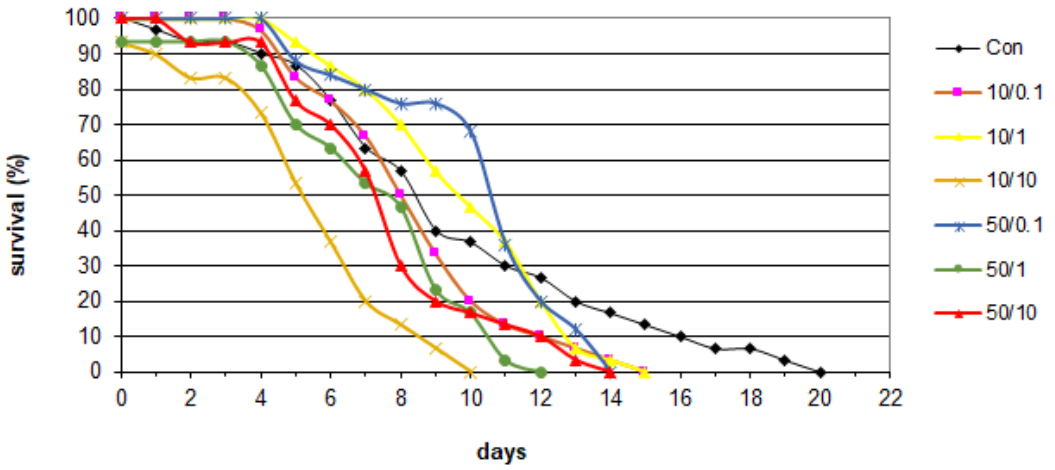

Figure 8. Cumulative survival of control mussels and mussels exposed to different concentrations of 10 µm and 50 µm PMMA particles through days

* Black – Control group; Pink - 10 µm PMMA low conc.; Yellow - 10 µm PMMA mid conc.; Orange - 10 µm PMMA mid conc.; Blue - 50 µm PMMA low conc.; Green - 50 µm PMMA mid conc.; Red - 50 µm PMMA high conc.

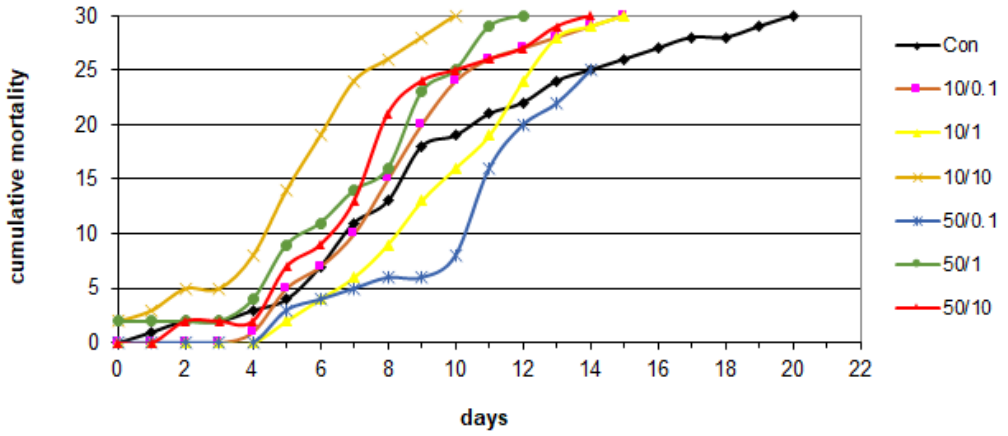

Figure 9. Cumulative mortality of control mussels and mussels exposed to different concentrations of 10 µm and 50 µm PMMA particles through days

* Black – Control group; Pink - 10 µm PMMA low conc.; Yellow - 10 µm PMMA mid conc.; Orange - 10 µm PMMA mid conc.; Blue - 50 µm PMMA low conc.; Green - 50 µm PMMA mid conc.; Red - 50 µm PMMA high conc.

Table 4. represent LT_{50} regression analysis of control mussels and mussels exposed to different concentrations of 10 µm and 50 µm PMMA. It is seen that the lowest LT_{50} has mussels exposed to 10 mg/L of 10 µm PMMA (4.35), which confirms the lowest cumulative survival of 10 days. The biggest LT_{50} has mussels exposed to 0.1mg/L of 50 µm PMMA (9.55), which had a cumulative survival of 14 days.

Table 4. LT_{50} regression analysis of control mussels and mussels exposed to different concentrations of 10 µm and 50 µm PMMA particles.

Control	**10/0.1**	**10/1**	**10/10**	**50/0.1**	**50/1**	**50/10**	**LT_{50} REGRESSION ANALYSIS (TOXICOLOGIST-PROBIT)**
30.00	30.00	30.00	30.00	25.00	30.00	30.00	
8.32	**7.86**	**9.29**	**4.35**	**9.55**	**6.48**	**6.76**	Median survival time LT_{50}
6.47	7.38	8.80	2.98	8.37	too wide	5.47	95% fiducial limits for LT_{50} from
9.98	8.31	9.77	5.58	10.88	too wide	7.74	95% fiducial limits for LT_{50} to

* Con – Control group; 10/0.1, 1, 10 – 10 µm PMMA/ LOW, MID, HIGH concentration; 50/0.1, 1, 10 – 50 µm PMMA/ LOW, MID, HIGH concentration

4.3 HAEMOCYTES ANALYSES

Figure 10. shows total haemocyte count (THC) of control mussels (CON) and mussels exposed to 0.1mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 10 µm PMMA particles after 24, 48 and 72h. THC in the control group is lowest after 24h, after 48h THC is the highest and then decreasing after 72h. In mussels exposed to LOW concentration, THC is the lowest after 24h and highest after 48h of exposure. In mussels exposed to MID concentration, THC is highest after 24h of exposure and then it's reduced with time, but after 72h is slightly higher than after 48h. At HIGH concentration, THC is lowest after 48h and highest after 72h of exposure.

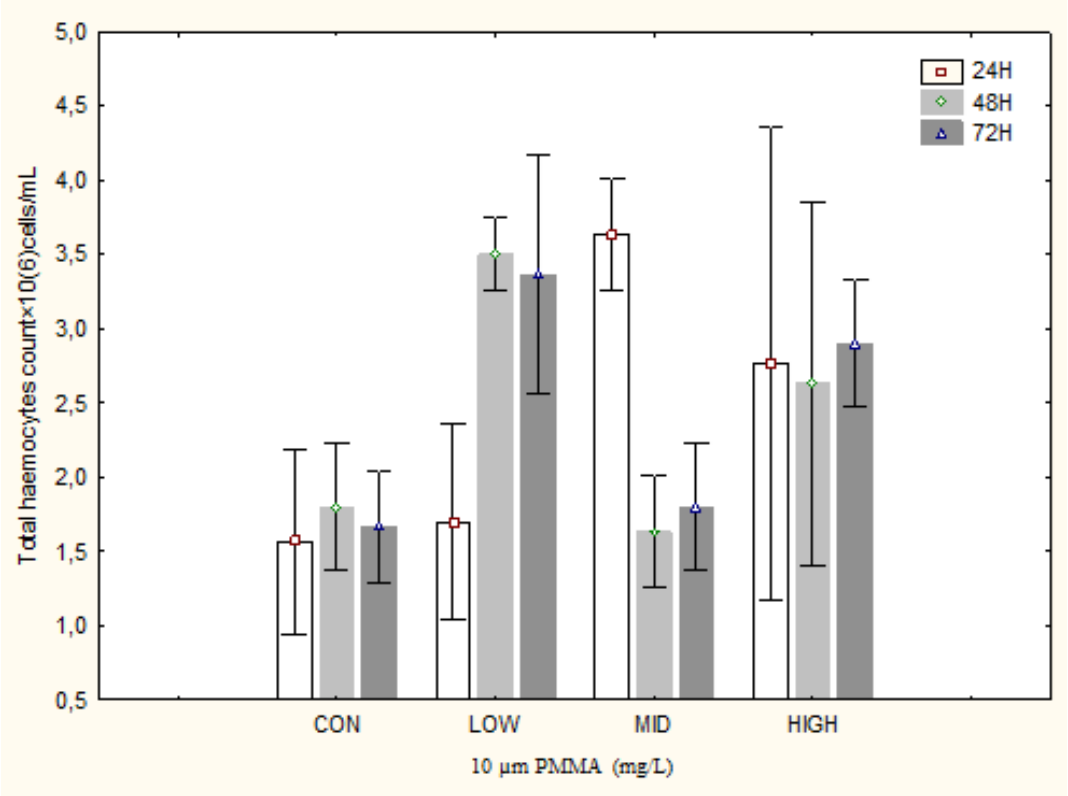

Figure 10. Total haemocytes count of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 10 µm PMMA particles after 24, 48 and 72h

The most significant impact of 10 µm PMMA on haemocytes is after 24h at MID concentration, which is proved with one-way ANOVA (SS=8.5166, p=**0.0004**). Effect is lower after 48h (SS=6.6358, p=**0.0001**). Effect of microplastic on haemocytes reduces with time, so the lower impact on mussels is after 72h (one-way ANOVA, SS=6.2333, p=**0.00002**) (Table 5).

Table 5. Statistical analyses (ONE WAY ANOVA, Statistica 9.0) of total haemocytes count exposed to 10 µm PMMA particles after 24, 48, and 72 h. Significants are bolded.

hour	SS	df	MS	F	p
24	8.5166	3	2.8388	19.9220	**0.0004**
48	6.6358	3	2.2119	28.8514	**0.0001**
72	6.2333	3	2.0777	44.524	**0.00002**

* SS – the sum of squares; df – the degrees of freedom; MS – the mean sum of squares; F – the *F*-statistic; p – the *p*-value

After 24h, there is a statistically significant difference in total haemocytes count between different concentrations of microplastic. Statistically, significant differences within different groups are calculated with post hoc Tukey HSD, Statistica 9.0 (Table 6). Statistically significant differences are shown between MID (p= 0.0008) and HIGH (p=0.0193) with CON, and MID (p=0.0012) and HIGH (p= 0.034) with LOW.

Table 6. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of total haemocytes count exposed to 10 µm PMMA particles after 24h. Significants are bolded.

The concentration of 10 µm PMMA	LOW	MID	HIGH
CON	0.0971	**0.0008**	**0.0193**
LOW		**0.0012**	**0.034**
MID			0.0868

After 48h, statistical differences are shown between LOW (p=0.0004) and HIGH (p=0.0256) with CON; MID (p=0.0003) and HIGH (p=0.0209) with LOW and MID and HIGH (p=0.0096) (Table 7).

Table 7. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of total haemocytes count exposed to 10 µm PMMA particles after 48h. Significants are bolded.

The concentration of 10 µm PMMA	LOW	MID	HIGH
CON	**0.0004**	0.8795	**0.0256**
LOW		**0.0003**	**0.0209**
MID			**0.0096**

After 72h, statistical differences are shown between CON and LOW (p=0.0002), CON and HIGH (p=0.0006), LOW and MID (p=0.0002) and MID and HIGH (p=0.0012) (Table 8).

Table 8. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of total haemocytes count exposed to 10 µm PMMA particles after 72h. Significants are bolded.

The concentration of 10 µm PMMA	LOW	MID	HIGH
CON	**0.0002**	0.8718	**0.0006**
LOW		**0.0002**	0.1097
MID			**0.0012**

Figure 11. shows total haemocyte count of control mussels (CON) and mussels exposed to 0.1mg/L (LOW), 1.0 mg/L (MID), and 10 mg/L (HIGH) concentration of 50 µm PMMA particles after 24, 48 and 72h. In the control group, THC was lowest after 24h. After 48h THC is the highest and then is decreasing after 72h. In mussels exposed to LOW concentration, THC is increasing gradually, after 24h of exposure THC is lowest and after 72h is the highest. In mussels exposed to MID concentration, the lowest THC is after 24h of exposure. After 48 and 72h THC is increasing and abundances are the same. At HIGH concentration, THC is lowest after 24h, THC is highest after 48h with a decrease after 72h.

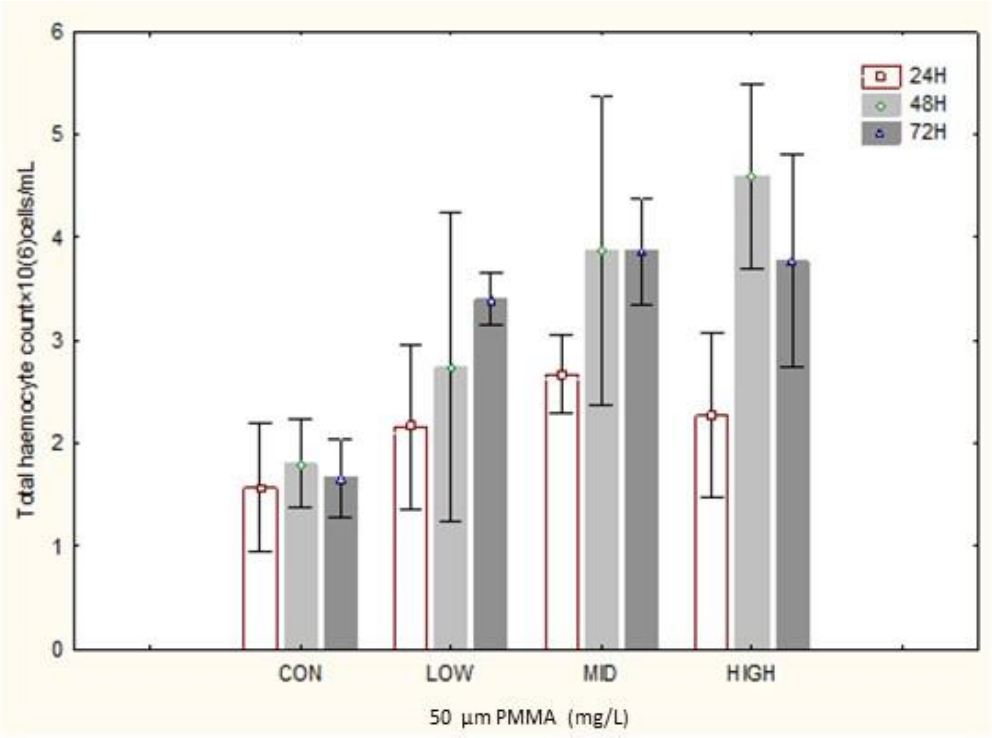

Figure 11. Total haemocytes count of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 50 µm PMMA particles after 24, 48 and 72h.

The biggest impact of 50 µm PMMA particles on THC is after 48h at HIGH concentration, which is proved with one-way ANOVA (SS= 13.7167, p=**0.0004**). The lowest impact was after 24h (one-way ANOVA, SS= 1.86, p=**0.0073**). The impact of microplastic particles reduces with time, so after 72h impact is lower than after 48h (SS= 9.4625, **p=0.00001**) (Table 9).

Table 9. Statistical analyses (ONE WAY ANOVA, Statistica 9.0) of total haemocytes count exposed to 50 µm PMMA particles after 24, 48, and 72 h. Significants are bolded.

hour	SS	df	MS	F	p
24	1.86	3	0.62	8.4545	**0.0073**
48	13.7167	3	4.5722	20.6266	**0.0004**
72	9.4625	3	3.1542	50.467	**0.00001**

* SS – the sum of squares; df – the degrees of freedom; MS – the mean sum of squares; F – the *F*-statistic; p – the *p*-value

After 24h, there is a statistically significant difference between total haemocytes count in different concentrations of microplastic. Statistically, significant differences within different groups are calculated with post hoc Tukey HSD, Statistica 9.0 (Table 10). Statistically significant differences are shown between CON and MID (p= 0.0048), CON and HIGH (p= 0.0527).

Table 10. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of total haemocytes count exposed to 50 µm PMMA particles after 24h. Significants are bolded.

The concentration of 50 µm PMMA	LOW	MID	HIGH
CON	0.0997	**0.0048**	**0.0527**
LOW		0.1867	0.9673
MID			0.336

After 48h, statistical differences are shown between CON and MID (p=0.003), CON and HIGH (p=0.0005), and LOW and HIGH (p=0.0056) (Table 11).

Table 11. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of total haemocytes count exposed to 50 µm PMMA particles after 48h. Significants are bolded.

The concentration of 50 µm PMMA	LOW	MID	HIGH
CON	0.1486	**0.003**	**0.0005**
LOW		0.0716	**0.0056**
MID			0.2972

After 72h, statistical differences are shown between CON and LOW (p=0.0003), CON and MID (p=0.0002), and CON and HIGH (p=0.0002) (Table 12).

Table 12. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of total haemocytes count exposed to 50 µm PMMA particles after 72h. Significants are bolded.

The concentration of 50 µm PMMA	LOW	MID	HIGH
CON	**0.0003**	**0.0002**	**0.0002**
LOW		0.1805	0.3413
MID			0.9592

4.4 CELL VIABILITY

Figure 12. shows cell viability of control mussels (CON) and mussels exposed to 0.1mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) of 10 µm PMMA after 24, 48 and 72h. In the control group, the highest cell viability was after 48h with a decrease after 72h. In mussels exposed to LOW concentration, cell viability is lowest after 24h. Cell viability is the highest after 48h with a decrease after 72h. In mussels exposed to MID concentration, the highest cell viability is after 24h of exposure, after 48 and 72h cell viability is decreasing. At HIGH concentration, the highest cell viability is after 24h of exposure.

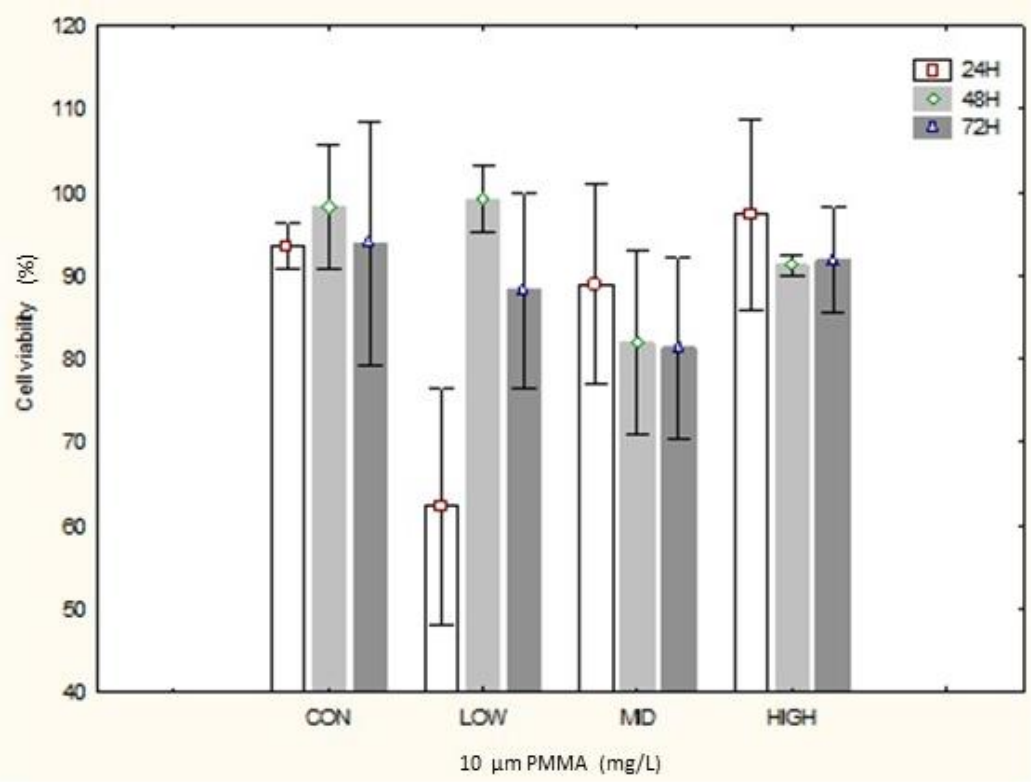

Figure 12. Cell viability of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 10 µm PMMA particles after 24, 48 and 72h

The biggest impact of 10 µm PMMA particles on cell viability of mussels is after 24h at LOW concentration (one-way ANOVA, SS= 2273.12; p= 0.00004). Impact on cell viability reduces with time, after 48h (SS= 570.8; p= 0.0002) and after 72h impact is the lowest (SS= 275.71; p= 0.0402) (Table 13).

Table 13. Statistical analyses (ONE WAY ANOVA, Statistica 9.0) of cell viability exposed to 10 µm PMMA particles after 24, 48, and 72 h. Significants are bolded.

hour	SS	df	MS	F	p
24	2273.12	3	757.71	38.329	**0.00004**
48	570.8	3	190.3	23.67	**0.0002**
72	275.71	3	91.9	4.464	**0.0402**

* SS – the sum of squares; df – the degrees of freedom; MS – the mean sum of squares; F – the *F*-statistic; p – the *p*-value

After 24h, there is a statistically significant difference between cell viability exposed to different concentrations of 10 µm PMMA particles. Statistically significant differences within different groups are calculated with post hoc Tukey HSD, Statistica 9.0 (Table 14). Statistically significant differences are shown between CON and LOW (p= 0.0003), LOW and MID (p= 0.0005) and LOW and HIGH (0.0002).

Table 14. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of cell viability exposed to 10 µm PMMA particles after 24h. Significants are bolded.

The concentration of 10 µm PMMA	LOW	MID	HIGH
CON	**0.0003**	0.6146	0.7238
LOW		**0.0005**	**0.0002**
MID			0.1753

After 48h, statistical differences are shown between CON and MID (p=0.0006), LOW and MID (p=0.0005), LOW and HIGH (p=0.0379) and MID and HIGH (p=0.0163) (Table 15).

Table 15. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of cell viability exposed to 10 µm PMMA particles after 48h. Significants are bolded.

The concentration of 10 µm PMMA	LOW	MID	HIGH
CON	0.9832	**0.0006**	0.0625
LOW		**0.0005**	**0.0379**
MID			**0.0163**

After 72h, statistical differences are shown between CON and MID (p=0.0382) (Table 16).

Table 16. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of cell viability exposed to 10 µm PMMA particles after 72h. Significants are bolded.

The concentration of 10 µm PMMA	LOW	MID	HIGH
CON	0.463	**0.0382**	0.9464
LOW		0.3145	0.7578
MID			0.0817

Figure 13. shows cell viability of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 50 µm PMMA particles after 24, 48 and 72h. In the control group, the lowest cell viability was after 24h. The highest cell viability was after 48h with a decrease after 72h. In mussels exposed to LOW concentration, cell viability is lowest after 24h. Cell viability is the highest after 48h with a small decrease after 72h. In mussels exposed to MID concentration, cell viability after 24 and 48h of exposure are almost the same, after 72h cell viability is the highest. At HIGH concentration, the lowest cell viability is after 24h of exposure and increase after 48h.

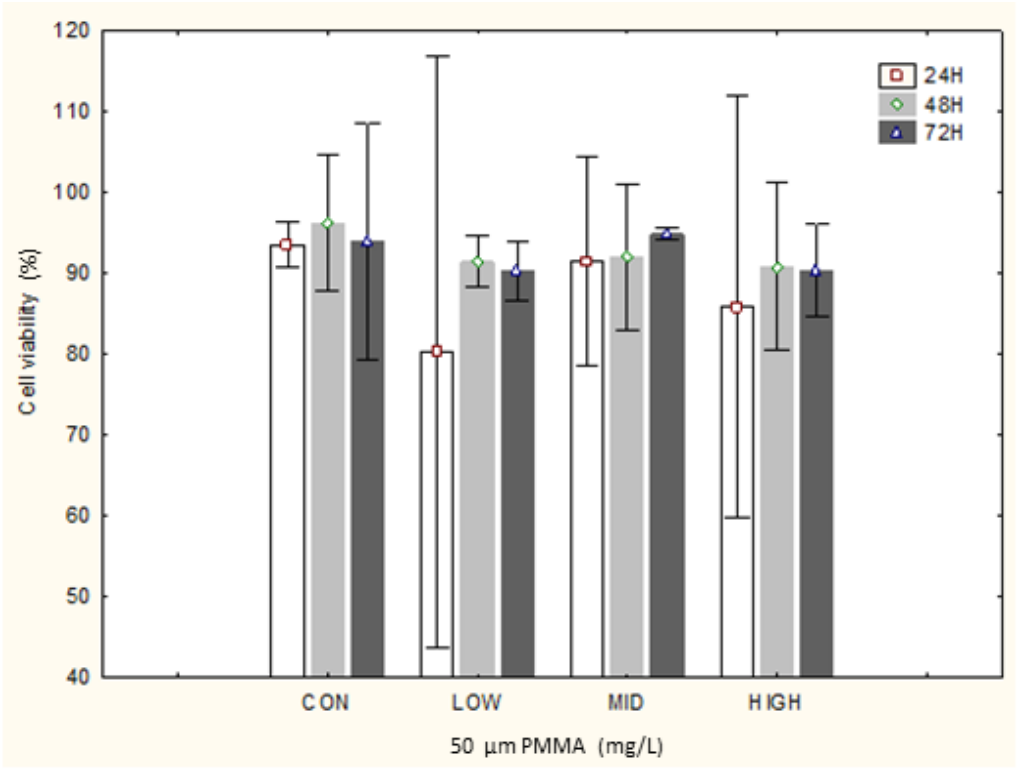

Figure 13. Cell viability of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 50 µm PMMA particles after 24, 48 and 72h

The most significant impact of 50 µm PMMA particles on cell viability of mussels is after 24h (one-way ANOVA, SS= 321.25). The effect on cell viability reduces with time, after 48h (SS= 53.3) and after 72h, the impact is the lowest (SS= 50.8) (Table 17). One-way ANOVA didn't show any statistical differences, so other statistical tests didn't been made.

Table 17. Statistical analyses (ONE WAY ANOVA, Statistica 9.0) of cell viability exposed to 50 µm PMMA particles after 24, 48, and 72 h.

hour	SS	df	MS	F	p
24	321.25	3	107.08	0.3827	0.7684
48	53.3	3	17.8	1.644	0.2548
72	50.8	3	16.9	1.603	0.2635

* SS – the sum of squares; df – the degrees of freedom; MS – the mean sum of squares; F – the *F*-statistic; p – the *p*-value

4.5 MULTINUCLEATED HAEMOCYTES

The occurrence of haemocyte vacuolation (Figure 14) known as ‘multi nuclei’ (multinucleated haemocytes, micronuclei, and binucleated cells) and deformation of the nucleus of haemocytes was observed in exposed mussels.

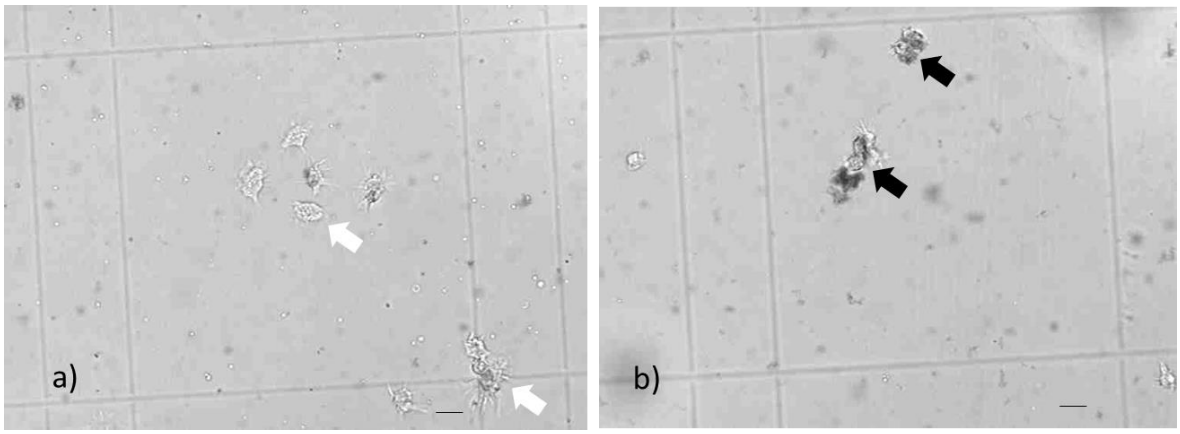

Figure 14. a) normal haemocytes (white arrow) from control mussel and b) malformed haemocytes (black arrow) from mussel exposed to 50 µm PMMA particles with vacuolation and deformed nucleus (400X). Blackline measures 10 µm

Figure 15. shows the number of vacuolized cells of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 10 µm PMMA particles after 24, 48 and 72h. In the control group, the lowest number of vacuolized cells was detected after 48h. The number of vacuolized cells is increasing through time so the most number of vacuolized cells is seen after 72h in mussels exposed to LOW concentration. At MID concentration, the highest number of the vacuolized cell was detected after 48h, with a decrease after 72h. After 24h, the presence of vacuolized cells was the lowest. The highest number of vacuolized cells was after 48h and the lowest after 72h at HIGH concentration.

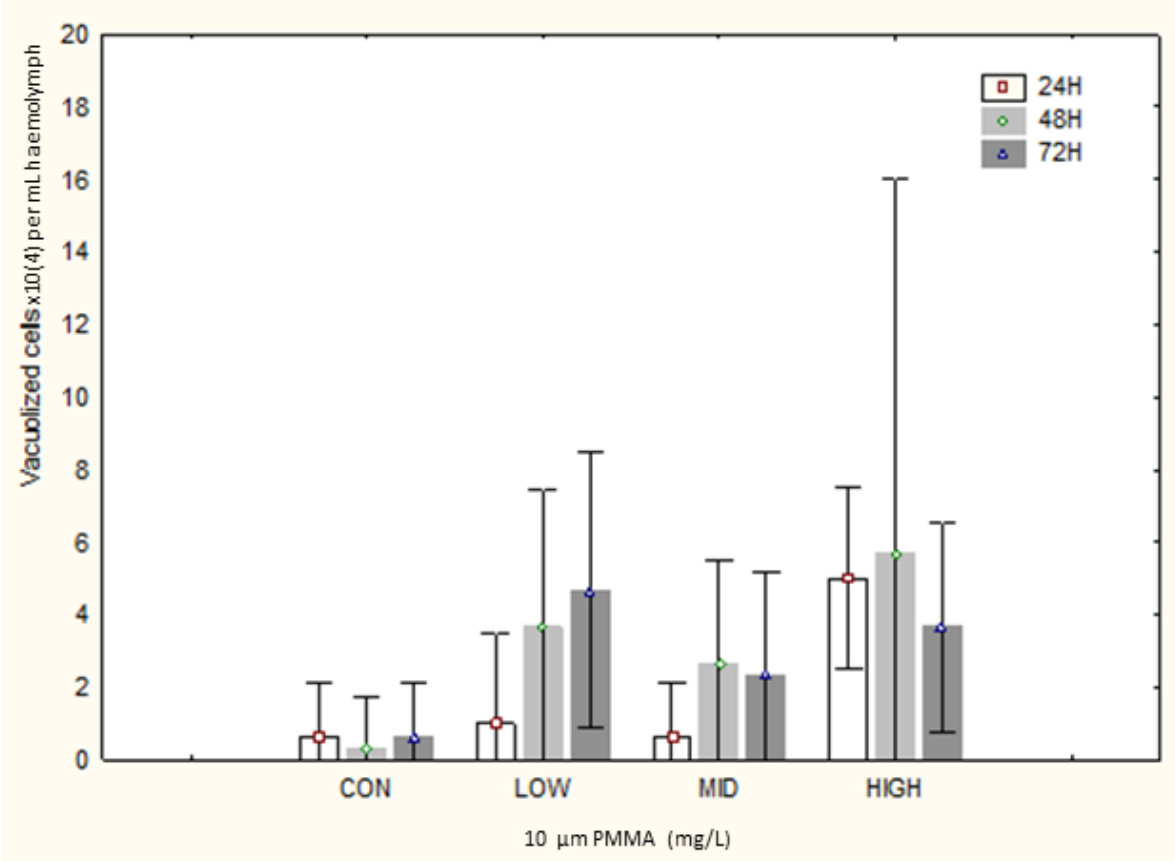

Figure 15. Number of vacuolized cells of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 10 µm PMMA particles after 24, 48 and 72h

The statistically significant impact of microplastics 10 µm PMMA on vacuolized cells of mussels is seen after 24h (one-way ANOVA, SS=40.333, p=0.0004), and 72h (one-way ANOVA, SS=20, p=0.0139) (Table 18).

Table 18. Statistical analyses (ONE WAY ANOVA, Statistica 9.0) of vacuolized cells exposed to 10 µm PMMA particles after 24, 48, and 72 h. Significants are bolded.

hour	SS	df	MS	F	p
24	40.333	3	13.4444	20.1666	**0.0004**
48	44.25	3	14.75	2.7656	0.1111
72	20	3	9	6.75	**0.0139**

* SS – the sum of squares; df – the degrees of freedom; MS – the mean sum of squares; F – the *F*-statistic; p – the *p*-value

After 24h, there is a statistically significant difference between vacuolized cells exposed t0 different concentrations of 10 µm PMMAparticles. Statistically significant differences within different groups are calculated with post hoc Tukey HSD, Statistica 9.0 (Table 19).

Statistically significant differences are shown between CON and HIGH (p= 0.001), LOW and HIGH (p=0.0016), and MID and HIGH (p=0.001).

Table 19. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of vacuolized cells exposed to 10 µm PMMA particles after 24h. Significants are bolded.

The concentration of 10 µm PMMA	LOW	MID	HIGH
CON	0.9568	1	**0.001**
LOW		0.9568	**0.0016**
MID			**0.001**

After 48h, one-way ANOVA didn't show any statistical differences, so other statistical tests didn't been made.

After 72h, statistical differences are shown between CON and LOW (p=0.0121) and CON and HIGH (p=0.0515) (Table 20).

Table 20. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of vacuolized cells exposed to 10 µm PMMA particles after 72h. Significants are bolded.

The concentration of 10 µm PMMA	LOW	MID	HIGH
CON	**0.0121**	0.3533	**0.0515**
LOW		0.1393	0.7211
MID			0.5253

Figure 16. represents the number of vacuolized cells of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 50 µm PMMA particles after 24, 48 and 72h. In the control group, the number of vacuolized cells was the lowest after 48h. After 24 and 72h number was almost the same but, in both cases, higher than after 48h. It is seen that the number of vacuolized cells was lowest after 24h and the highest after 72h in all 3 groups of mussels exposed to PMMA. The highest increase in the number of vacuolized cells was detected between 24 and 48h at mussels exposed to the HIGH concentration.

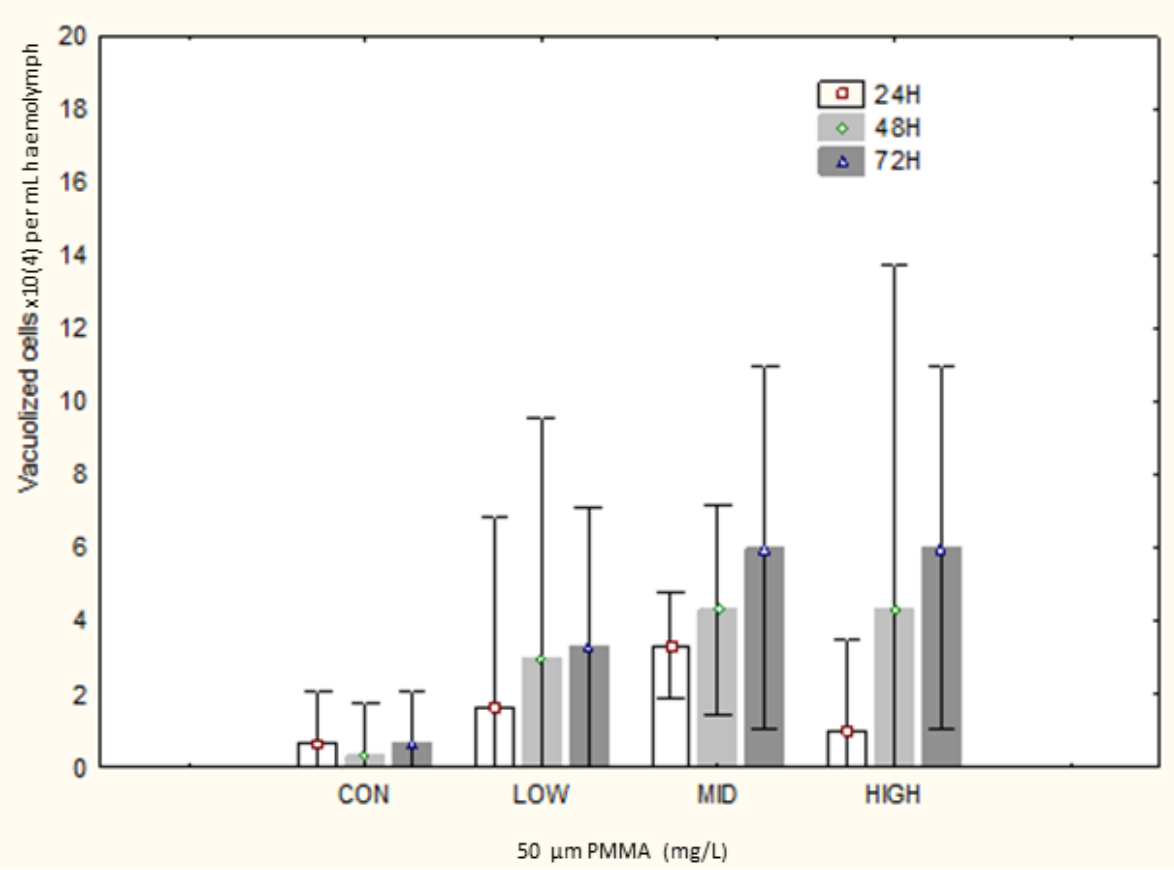

Figure 16. Number of vacuolized cells of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 50 µm PMMA particles after 24, 48 and 72h

The most significant impact of 50 µm PMMA particles on vacuolized cells of mussels is seen after 72h (one-way ANOVA, SS= 58.6667, p=0.011). After 24h (p= 0.1075) and 48h (p=0.2155), one-way ANOVA didn't show any statistical differences, so other statistical tests have not been made (Table 21).

Table 21. Statistical analyses (ONE WAY ANOVA, Statistica 9.0) of vacuolized cells exposed to 50 µm PMMA particles after 24, 48, and 72 h. Significants are bolded.

hour	SS	df	MS	F	p
24	12.6666	3	4.2222	2.8148	0.1075
48	32	3	10.6667	1.855	0.2155
72	58.6667	3	19.5556	7.3333	**0.011**

* SS – the sum of squares; df – the degrees of freedom; MS – the mean sum of squares; F – the *F*-statistic; p – the *p*-value

After 72h, there is a statistically significant difference between the different concentrations of 50 µm PMMA particles. Statistical significant differences within various groups are calculated with post hoc Tukey HSD, Statistica 9.0 (Table 21). Statistical differences are shown between CON and MID (p=0.0382) and CON and HIGH (p=0.0167) (Table 22).

Table 22. Statistical analyses (post hoc Tukey HSD, Statistica 9.0) of vacuolized cells exposed to 50 µm PMMA particles after 72h. Significants are bolded.

The concentration of 50 µm PMMA	LOW	MID	HIGH
CON	0.2642	**0.0382**	**0.0167**
LOW		0.2642	0.2642
MID			1

4.6 MICROPLASTICS IN TISSUES

Both particle sizes of PMMA (10µm and 50 µm) were observed in mussel haemolymph after 48 hours from exposure (Figure 17).

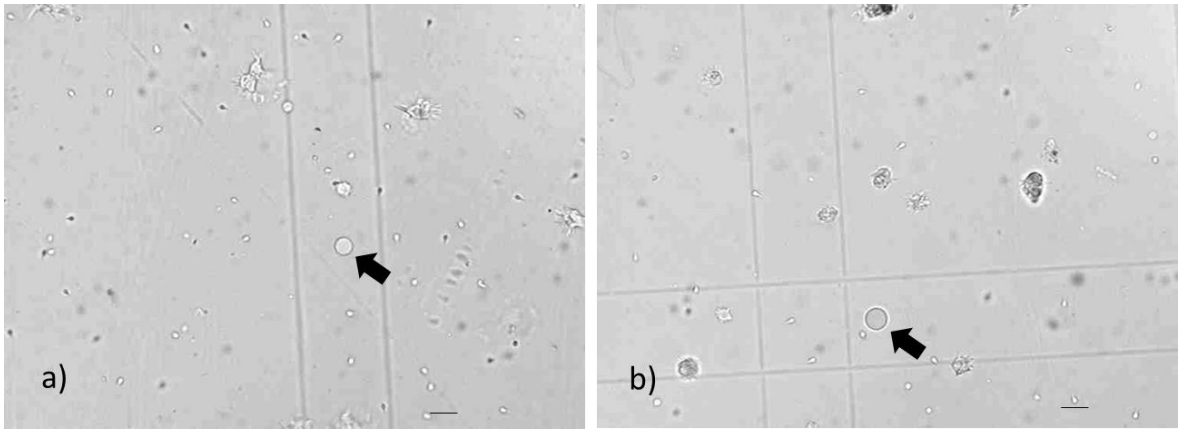

Figure 17. a) 10 µm PMMA particles (black arrow) in the haemolymph of mussel exposed to 0.1 mg/L and b) 50 µm PMMA particles (black arrow) from mussel exposed to 0.1 mg/L after 48h from exposure. (400X). Blackline measures 20 µm.

10 µm PMMA particles were detected in the mussel digestive gland and gills of exposed mussels, while 50 µm PMMA particles were not observed in mussel tissues. Figures 18. and 19. represent tubules of the digestive gland of mussels. The most concentration of particles of 10 µm PMMA was found in digestive tubules in mussels exposed to HIGH concentration (10 mg/L).

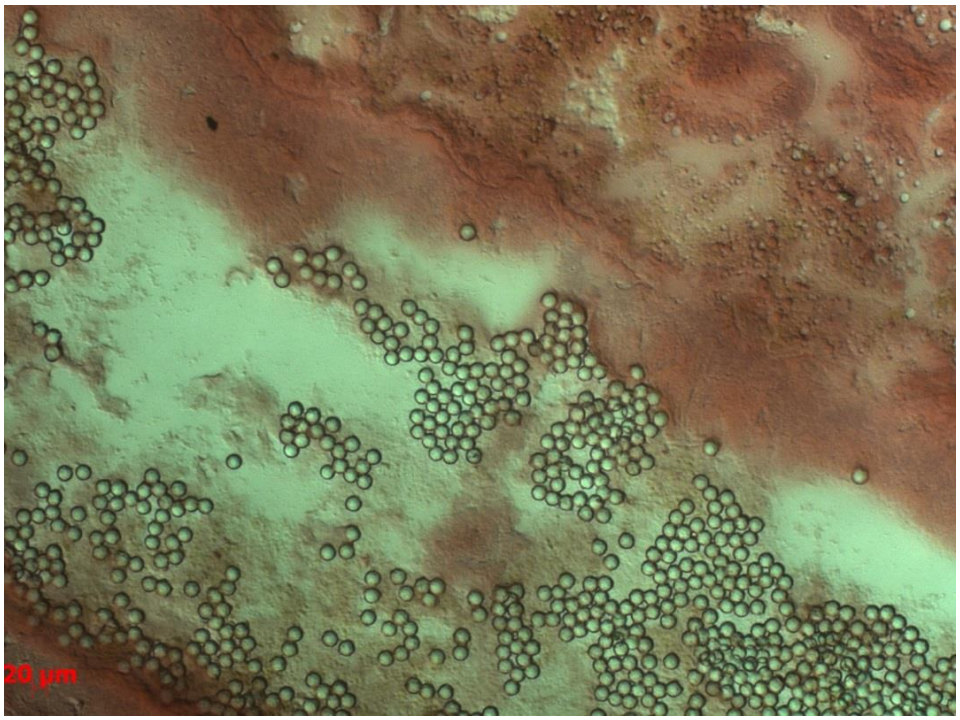

Figure 18. Particles of 10 µm PMMA (10 mg/L) in the digestive gland (400x)

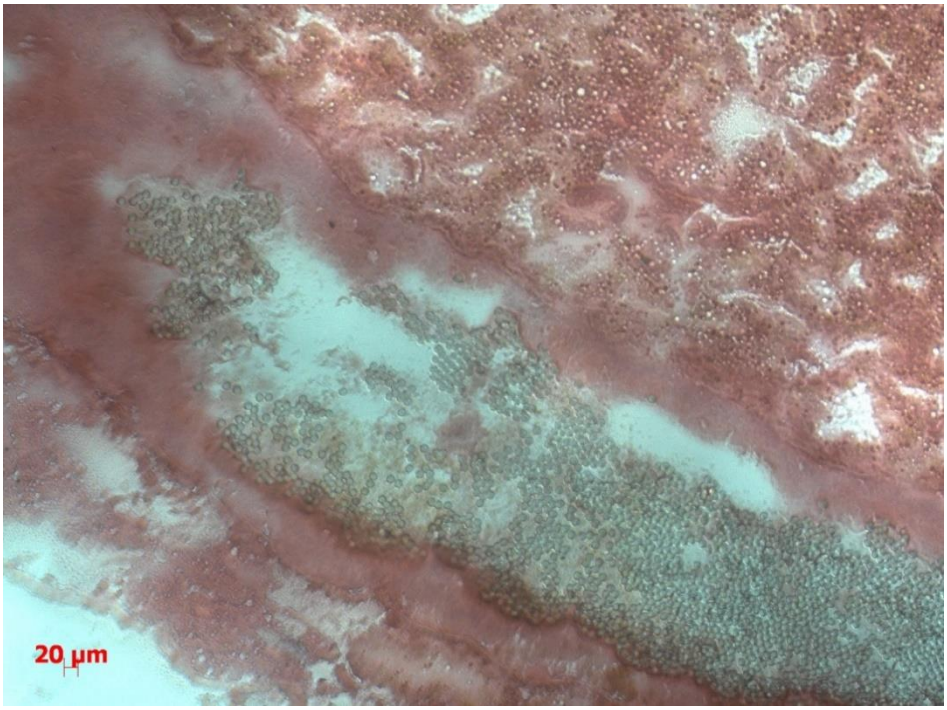

Figure 19. Particles of 10 µm PMMA (10 mg/L) in the digestive gland (100X)

Figure 20. represents the number of MPs in the digestive gland section of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID), and 10 mg/L (HIGH) concentration of 10 µm PMMA particles. Microplastic particles were most abundant at HIGH concentration. Smallest abundance was detected at LOW concentration. The control group wasn't been exposed to MPs.

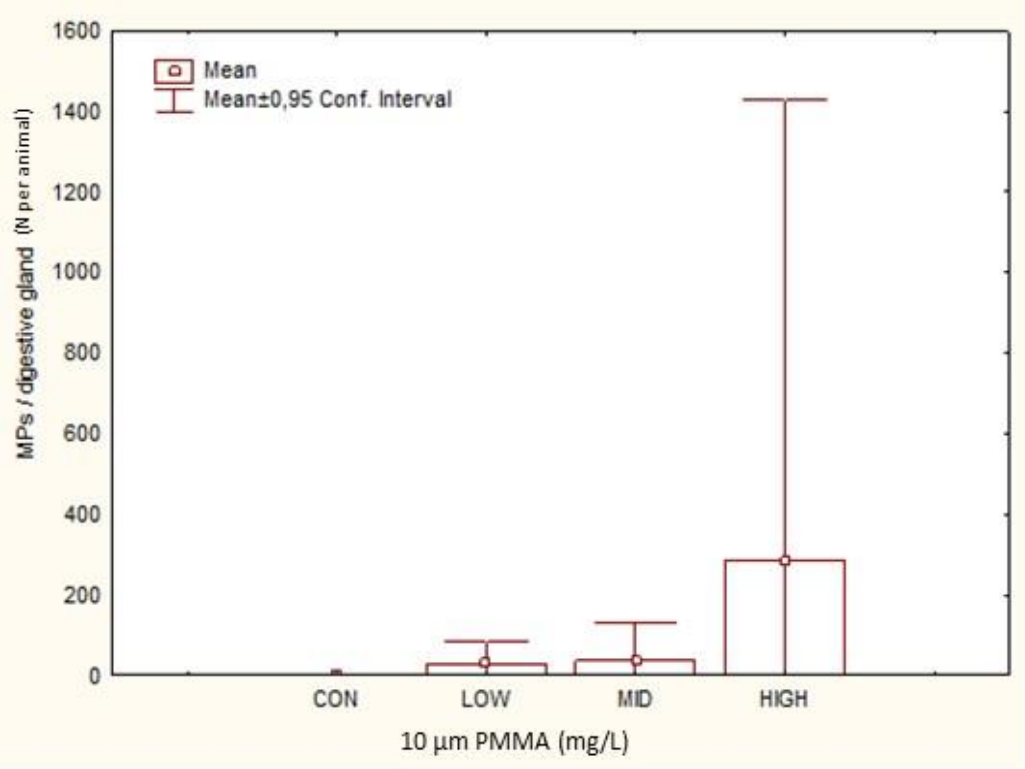

Figure 20. Number of MPs in digestive gland section of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 10 µm PMMA particles

10 µm PMMA particles were found in a tissue section of mussels gills. Figures 21. and 22. represent a tissue section of mussels exposed to MID (1.0 mg/L) and HIGH concentration (10 mg/L) of MPs. The particle of 10 µm PMMA was found in a gill section in mussel exposed to MID concentration (1.0 mg/L) (Figure 21) and HIGH concentration (10 mg/L) (Figure 22).

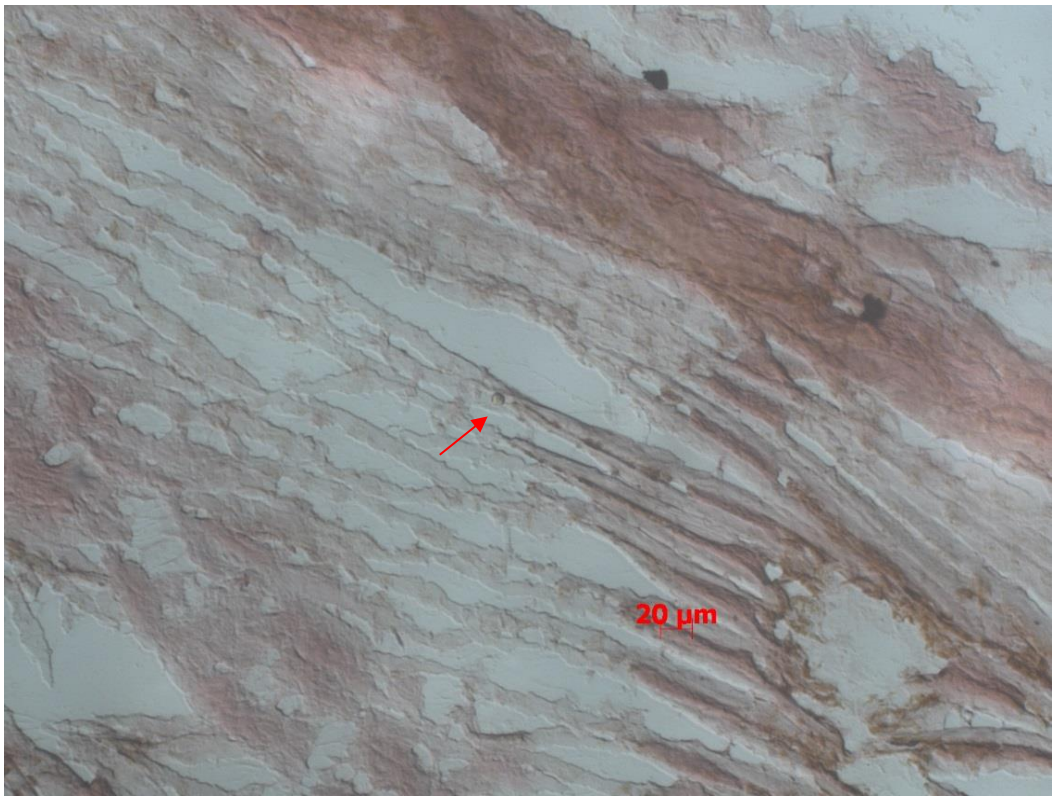

Figure 21. The particle of 10 µm PMMA (1.0 mg/L) on gills (100x)

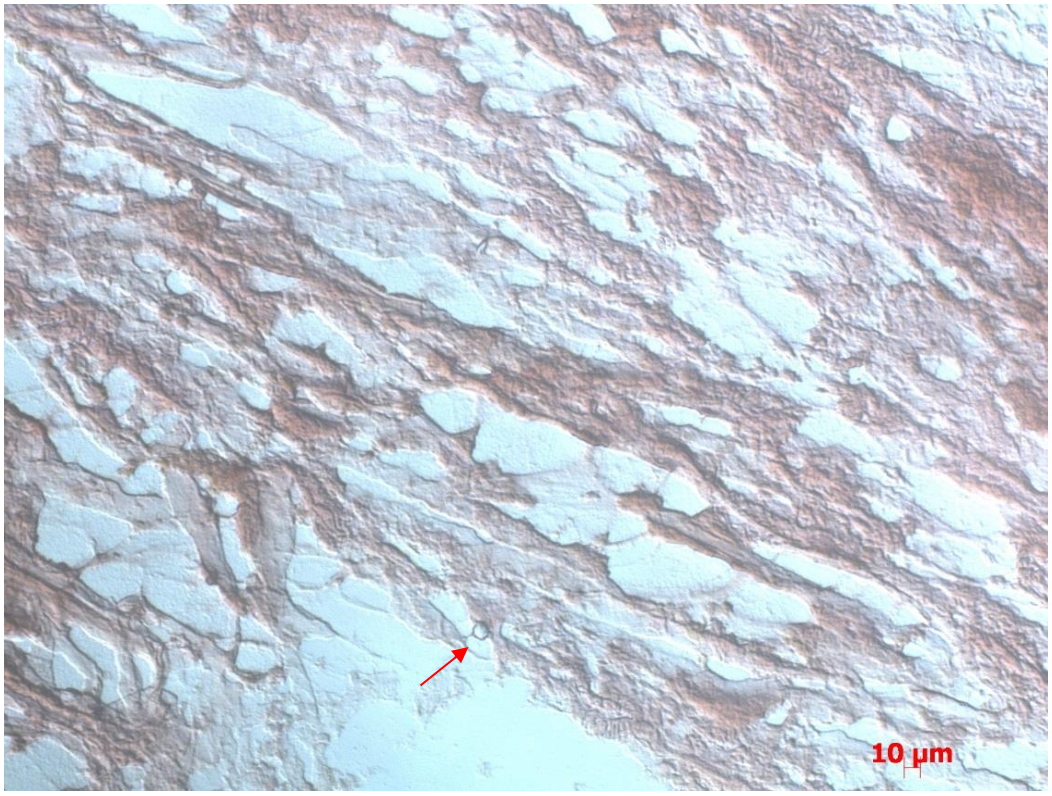

Figure 22. The particle of 10 µm PMMA (10 mg/L) on gills (100x)

Figure 23. represents the number of MPs in gills section of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID), and 10 mg/L (HIGH) concentration particles of 10 µm PMMA. Microplastic particles in gills were most abundant at LOW and HIGH concentration. The smallest number of MPs were detected at MID concentration when compared to others.

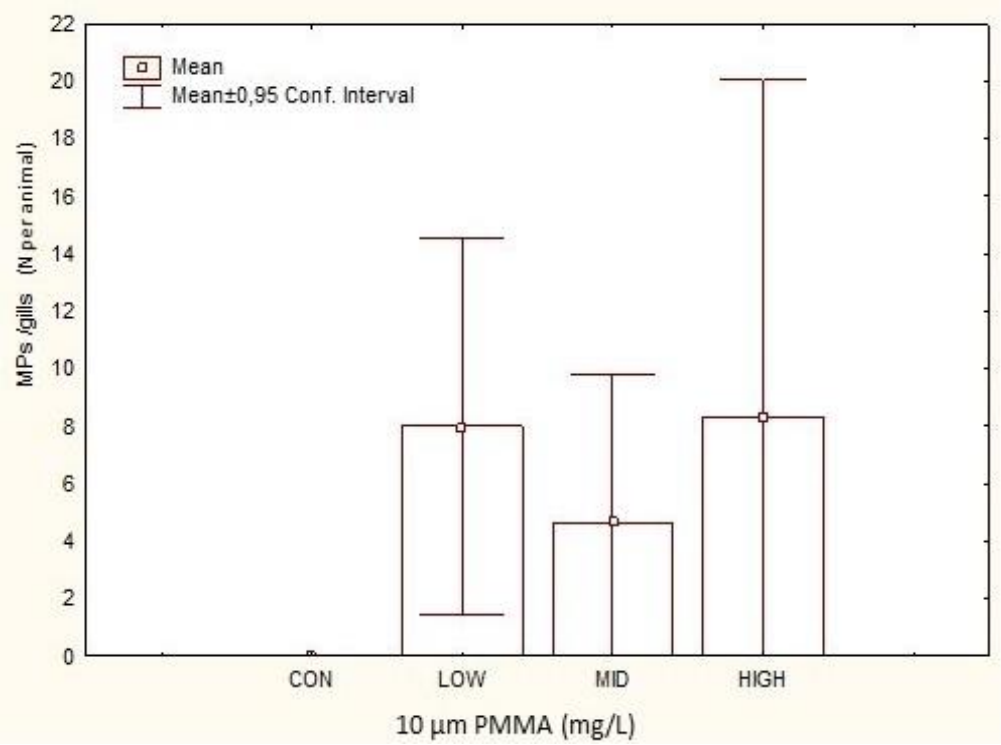

Figure 23. Number of MPs in gills section of control mussels (CON) and mussels exposed to 0.1 mg/L (LOW), 1.0 mg/L (MID) and 10 mg/L (HIGH) concentration of 10 µm PMMA particles

Non-parametric Kruskal – Wallis test was not detected in the statistical difference in the number of PMMA particles between mussel samples (Table 23).

Table 23. Kruskal – Wallis test of statistical difference in the number of PMMA particles in mussel digestive glands and gills.

	N	H	p
Digestive glands 10 µm	12	5.1147	0.1636
Gills 10 µm	12	7.5559	0.0561

* N- total number of values, H - approximately chi-square distributed, p - the *p*-value

5 DISCUSSION

Marine plastics can have significant ecological impacts. The impacts of macroplastics on biota are best known, but the impact of microplastic is much more difficult to quantify and remains a knowledge gap (Li et al., 2019). The Mediterranean mussel *Mytilus galloprovincialis* is widely distributed in urbanized coastal environments thus likely being exposed to MPs sources for its entire life cycle, it is also a species of high ecological and commercial importance and worldwide utilized as sentinel organism of pollution in coastal marine environments (Viarengo et al., 2007; Li et al., 2019). In this context, the effects of two-particle sizes (10 μm and 50 μm) of PMMA in low (0.1 mg/L), medium (1.0 mg/L), and high (10.0 mg/L) concentrations on mussel *M. galloprovincialis* has been investigated after a 72h exposure.

This experimental study aims to investigate if the PMMA particles are taken up into the mussel gills and transported to the haemolymph and digestive system. MPs can be taken up over the digestive surface of mussels by endocytosis and granulocytomas and then transferred to lysosomes and circulatory system or eliminated as pseudo faces particles (Browne et al., 2008; von Moos et al., 2012; Avivo et. al., 2015; Beyer et. al., 2017; Qu et. al., 2018). In this study, smaller particles (10 μm) are easily taken up into the tissues, they enter through gills, accumulated in the digestive gland, and transported to the haemolymph causing the effect on mussel health. As previously proved, smaller particle size can translocate from the gut cavity to the haemolymph and inside the haemolymph (Browne et al., 2008). Van Cauwenberghe et al. (2015) demonstrated that only microplastics of the smallest size (10μm) was detected in mussels although three sizes (10 μm, 30 μm, 90 μm of microplastics were used in the exposure experiment. This is proven in this study, larger particles (50 μm) were not found in the tissue of gills and digestive gland. Although the larger particles cannot penetrate the tissues slightly as quickly, they were found in the haemolymph. The results obtained in this study provide the first proof of principle that PMMA particles (10 and 50 μm) are taken up into haemolymph of *M. galloprovincialis.*

Mussel health status was evaluated by measuring the condition index (CI) and the survival test of mussels in the air (SOS test) after 72h exposure of PMMA particles. CI of mussels exposed to 10 μm PMMA particles statistically decreased with an increased concentration of MPs. CI of mussels exposed to 50 μm PMMA particles statistically lowest at concentration 0.1 mg/L, but CI at highers concentrations are significantly decreased in compared to CI of control mussels. The effects of smaller PMMA particle size (10 μm) has a greater influence on mussel condition indices than larger PMMA particle size (50 μm). The CI is used as an ecophysiological measure of the health status of animals (Kovačić et al., 2016). The CI can be affected by an environmental condition such as salinity, temperature, food availability, and endogenous factors (reproductive cycle) (Hamer et al.,

2008; Pavičić – Hamer et al., 2016). The CI measured in this study is similar to one measured in Kanduč et al. (2018) for Lim Bay in spring. They observed a higher CI of mussels from polluted locations than farmed mussels, which suggests that the physiological conditions for mussel growth are similar at both locations. In other words, mussels have good defense mechanisms against pollutants. In an experiment by von Moos et al. (2012) no effect was observed on the CI of mussels after HDPE MPs exposure. The results of this study provide that higher concentrations of PMMA particles had a more significant impact on mussel health.

"Stress on stress" (SOS) test is a physiological biomarker used for measuring the ability of mussels to survive exposure to air. In this test, individuals are exposed to the air that has already experienced the effects of stressors such as heavy metals, organic chemicals, microplastics (Thrush et al., 2008; Hewitt and Thrush, 2009; Andrade et. al., 2017). In this study, the test was performed to achieve general stress conditions in exposed mussels. The SOS test showed that mussels exposed to HIGH concentrations (10 mg/L) of 10 µm PMMA particles lived significantly shorter than mussels in the control group. LT_{50} of exposed mussels was the lowest at the HIGH concentration of 10 µm PMMA particles. In mussels exposed to a LOW (0.1 mg/L) and MID (1 mg/L) concentration of 10 µm PMMA, there was no statistically significant difference in LT_{50}. At mussels exposed to 50 µm PMMA particles in concentrations of 1 mg/L and 10 mg/L caused a shorter survival time compared to the control group, but this time is still slightly higher than mussels exposed to the HIGH concentration of 10 µm PMMA particles.

Haemocytes are macrophages that have a key role in the immune defense of mussels. They have an important role in defense against parasites, pathogens, pollutants (Pagano et al., 2017). Haemocytes circulate in haemolymph throughout the animal and the major organs, so there is a possibility that microplastic could be transported to the tissues of organs. In this study was observed that the HIGH concentration of PMMA particles has a constant increase of total haemocytes count (THC) relative to the control group after 24, 48, and 72 h. At MID concentration, after 24h, a decrease in THC was observed relative to the control group. At LOW concentration, after 24h, the organism wasn't ready enough to fight against MPs, but after 48 and 72h the immune response was stimulated to actively fight the microplastic particles found in the haemolymph. Because mussels are highly sensitive organisms, a significant finding in this experiment is that already low concentration of PMMA particles after 48 and 72h have shown a great response and influence of the organism to defend itself. It is important to say that the experiment was performed in May, following a spawning period, after which the mussels were ready for normal physiology. In other investigations, different kind of microplastics was also found in the haemolymph. Browne et al. (2008) detect the presence of polystyrene particles (3.0 and 9.6 µm) in the haemolymph and haemocytes of *Mytilus edulis* after three days. 3 µm microspheres were detected in

significantly higher amounts that 9.6 µm microspheres. The particles were still present in the haemolymph after 48 days. Franzellitti et al. (2014) expose *M. galloprovincialis* to 3 and 45µm PS-MPs for four days at 1 and 10 particle/ml. Particles were detected in the haemolymph in all treated mussels. Values were significantly different from the control group and treated mussels to 3µm (1 and 10 particle/ml) and 45µm (10 particle/ml). Our study confirms that exposure to MPs induced a significant increase in the haemocytes formation, thus increase are correlated with the concentration of PMMA particles with the time of exposure.

Cell viability is the quantification of the number of live cells and is usually expressed as a percentage of the total cell number (King, 2000). Assay to measure cell viability is used to monitor the response and health of cells in the organisms after treatment with the various toxicant. In this study has been observed that already LOW concentration (0.1 mg/L) of PMMA particles after 24h causes the highest decrease in cellular viability in both particle sizes of microplastics relative to the control group. Hoher er al. (2012) found a negative correlation between cell viability and vacuolation of haemocytes. They suggested the vacuolation of haemocytes as a marker of reduced cell viability. This correlation in our experiment is best seen after 72h at mussels exposed to 50 µm PMMA particles. Cell viability is decreasing as the concentration of particles increases, while the vacuolation of haemocytes increases to the highest particle concentration. Pagano et al. (2017) in treatment with other toxicants mussel *M. galloprovincialis,* also showed mortality of haemocytes. In treatment with zinc chloride and cadmium chloride, after 24h, no changes were seen. Zinc chloride after 72h and 7 days at concentrations of 0.5 and 1 mg/m, showed significant mortality compared to control (Pagano et al., 2017). Our results showed a significant impact of both PMMA particles on cell viability of mussels is after 24h, the effect on cell viability reduces with time.

In this study by histological analysis, the presence of 10 µm PMMA particles were detected on gills and digestive glands of exposed mussels. The most significant abundance of 10 µm PMMA was found in digestive tubules in mussels exposed to HIGH particle concentration (10 mg/L). At exposure to smaller particles, the abundance of MPs increases as the concentration increases. An interesting observation is that 50 µm PMMA particles were not found in tissues. Browne et al. (2008) found that mussel *M. edulis* exposed to polystyrene particles (3.0 and 9.6 µm) had accumulated particles in the gut cavity and digestive tubules. Von Moos et al. (2012) detect the presence of high-density polyethylene (HDPE) (0-80 µm) in the intestine, in the lumina of primary and secondary ducts of digestive gland and endocytotic vacuoles of digestive epithelial cells of *M. edulis.* Particles were also found in the blood lacunae of the gills and the areas of lamellar junctions. Particles seen on gills indicate that they were trapped from the water column. Not only the mussels but also the crabs show the ingestion of microplastics. Shore crab *Carcinus maenas* exposed to 8 – 10

µm polystyrene microspheres, had detectable numbers of microspheres on their gills (Watts et al., 2014). The first proof of the harmful effect of PMMA on sea urchin *Sphaerechinus granularis* was found by Trifuoggi et al. (2019). PMMA exposure increased developmental defects and microplastic uptake in plutei. Embryo exposure resulted in cytogenetic abnormalities (increased mitotic aberration) while a decrease of fertilization success was observed following sperm exposure. Overall, developmental, cytogenetic, and genotoxic effects were observed in *S. granularis* early life stages exposed to PMMA. The size of mussels also affects the ingestion of microplastics. Brate et al. (2018) found a significant positive correlation between the mussel body and the number of ingested particles. Larger mussels are prone to contain more microplastic particles than smaller sized mussels. Plastic pollution not only affects adult stages but has an impact on planktonic larvae also. Rist (2019) found that larvae of *M. edulis* ingest plastic particles of nano- and micro-scale. It was noted that larvae ingested a higher amount of 2 µm polystyrene beads than 100 nm. Exposure was not affected by larval growth, but abnormally developed larvae increased after exposure to polystyrene beads. Further research on the effect of 50 µm PMMA particles on tissues of *M. galloprovincialis* is needed after longer experimental exposure.

Data reported in this study showed that mussel can effectively ingest and accumulate PMMA particles, suggesting their potential role as plastic primary consumers in marine ecosystems. The present investigations were performed using PMMA particles in controlled laboratory conditions; thus, observed responses might not necessarily reproduce those occurring in natural systems, where organisms are simultaneously exposed to a broad range of structurally heterogeneous MPs of irregular shape/size and further associated stressors. Nevertheless, data highlight the vulnerability of bivalve towards MPs, providing baseline information for future investigation addressing their impact on marine ecosystems.

6 CONCLUSION

The widespread occurrence of microplastics (MPs) is becoming an increasing problem in the natural environment. MPs causing concern on the macro scale such as ingestion by marine animals and also at the microscale where small particles may impact directly on the cells, tissues, and organs. MPs in the marine environment have more subtle effects than bigger fragments because their size range is overlapping with that of particles ingested by filter-feeders. The results obtained in this study provide the first proof of principle that PMMA particles (polymethylmethacrylate, known as plexiglass) are taken up into the gills and digestive gland of the mussel *M*ytilus *galloprovincialis* and causes significant effects of mussel health. In this context, the effect of smaller particle sizes PMMA (10 μm) on mussel *M. galloprovincialis* has been observed more significant than the effect of bigger particles sizes (50 μm). However, all haemocyte analyses showed significant effects of both particle sizes PMMA on the exposed mussels by increasing total haemocytes counts (THC) and reducing cell viability with increasing concentration of MPs (0.1 mg/L, 1.0 mg/L and 10.0 mg/L). The results also noted a significant increase in levels of vacuolized haemocytes as a result of PMMA exposure (72h). This study provides proof of the principle that MPs are taken up into tissues, 10 μm PMMA particles are detected in gills and digestive glands of exposed mussels, causing physiological damages as decreased condition and fitness index. This study highlighted how PMMA particle size is important on different internalization mechanisms in organs, causing the key effect on main immunological cells.

7 POVZETEK V SLOVENSKEM JEZIKU

Plastika se danes vse pogosteje uporablja v industrijskih in potrošniških izdelkih. Posledica njene povečane proizvodnje in uporabe je nastajanje in odlaganje velikih količin plastičnih odpadkov, ki prekomerno obremenjujejo okolje. Kopičijo se tako na kopnem kot v vodnem ekosistemu. Zato obstaja povečano tveganje prehajanja mikroplastike, tj. drobnih delcev plastike v morski ekosistem. Prehajanje pa ne predstavlja problema samo za bioto, temveč ima lahko negativne učinke tudi na človeka.

Namen magistrske naloge je raziskati vpliv nizke, ekološko pomembne koncentracije mikroplastike in različne velikosti (10 µm in 50 µm) PMMA (polimetilmetakrilat) delcev na školjke. Modelni organizem je klapavica *Mytilus galloprovincialis* (Lamarck, 1819). V čistem morju raste precej počasi, medtem ko se njena rast v morju, bogatem s hranili (npr. pristanišča), hitro povečuje (Turk, 2011). Ker klapavice najpogosteje prebivajo v pristaniščih ob obali, kjer so številni industrijski izpusti, so izbrane kot vzorčni organizem magistrskega dela. Klapavica filtrira okoli 3 litre morske vode v eni uri, kar je 72 litrov na dan. Posledično se lahko v njihovem tkivu kopičijo različna okoljska onesnaževala. Cilj naloge je bil oceniti, ali se mikroplastični delci PMMA filtrirajo v klapavice in ali delci PMMA vstopajo v celice in povzročajo negativen učinek na njihovo fiziološko stanje. Fiziološka aktivnost in kondicija školjk po izpostavljenosti delcem PMMA se ocenjujeta s testom indeksa kondicije (IK) in SOS testom (stres-test). Celice se analizirajo s svetlobnim mikroskopom, medtem ko se vpliv na tkiva preučuje s histološkimi analizami.

V magistrski nalogi so postavljeni štiri glavni cilji:

a) preučiti vpliv mikrodelcev PMMA na klapavice

b) preučiti, ali izbran model mikrodelcev PMMA vstopa v organe klapavic

c) preveriti, ali se zaužiti mikrodelci PMMA prenašajo iz tkiv v cirkulatorni sistem

d) preučiti, ali bo zaužitje mikrodelcev PMMA vplivalo na hranjenje in fiziološko stanje školjk

Predpostavljene hipoteze so:

i. mikrodelci PMMA vstopajo v klapavice skozi škrge in prenesejo v hemolifo ter prebavni sistem

ii. mikrodelci PMMA se nabirajo v celicah in tkivih školjk, njihov učinek pa je odvisen od koncentracije in velikosti uporabljenih delcev

iii. manjši mikrodelci imajo na klapavice večji vpliv kot delci večjih premerov

iv. mikroplastični delci vplivajo na zdravstveno stanje klapavic

v. fiziološko stanje in fitnes klapavic se po izpostavljenosti mikrodelcem PMMA zmanjšujeta zaradi škodljivega vpliva mikroplastičnih delcev.

Raziskava bo pokazala maksimalno koncentracijo mikroplastičnih delcev PMMA, ki se lahko sprostijo v morsko okolje brez negativnih učinkov na klapavicee. Ugotovili bomo, ali je klapavica dovzetna za mikroplastične delce PMMA, v katerih tkivih se filtrira in kakšen je vpliv na kondicijo klapavice.

Metodologija

Klapavice so bile odvzete iz Limskega kanala in prenesene v bazene Centra za morske raziskave, Institut Ruđer Bošković v Rovinju. Morska voda v bazenih se dnevno menja in prezračuje. Vzorci klapavic so bili izpostavljeni različnim koncentracijam mikroplastičnih delcev PMMA. Po tridnevni izpostavljenosti sta bili izmerjeni njihova velikost in masa (za izračun indeksa kondicije).

Za določanje skupnega števila hemocitov je bilo s pomočjo igle, pritrjene na 1 ml brizgo odvzeto 500 µl hemolimfe iz aduktora posamezne klapavice. Število hemocitov v hemolimfi je bilo določeno s pomočjo hemocitometra (Neubayerjeva komora).

Vzorci tkiva prebavnih žlez in škrg, potrebni za histološko analizo, so bili odvzeti s škarjami, ohlajeni v tekočem dušiku in zamrznjeni v N-heksanu. Obdelani vzorci so bili shranjeni pri temperaturi -80 ºC, do priprave histoloških pripravkov. Pred kriosekcijo so bili vzorci vezani na nosilec mikrotoma in potopljeni v medij O.C.T. TM (Microm Inc. GmbH, Germany) (Kovačić, 2018). Vzorci tkiva so bili postavljeni na nosilec za kriotomijo (Zeiss Hyrax C 50, Microm GmbH, Germany). Nosilec je bil predhodno ohlajen na -30 ºC. Za vsak vzorec tkiva so bili pripravljeni 10 µm debeli rezani pripravki. Zamrznjeni prečni prerezi prebavnih žlez so bili postavljeni na predmetno stekelce, segreto na sobno temperaturo. Preparati so bili obarvani z raztopino hematoksilina in eozina (Sigma-Aldrich, USA) pri sobni temperaturi. Po barvanju so bili pripravki vstavljeni v glicerolsko želatino (Sigma – Aldrich, USA) in opazovani pod polarizacijskim mikroskopom.

Za izračun indeksa kondicije je bilo odvzetih 10 vzorcev klapavic iz vsakega bazena. IK je bil določen po delu Pavičić-Hamer i sur. (2016). S pomičnim merilom so bile izmerjene dolžina, širina in višina posamezne klapavice. Ločeno je bila stehtana masa školjke, masa školjke brez vode ter masa mokrega tkiva in ljuštura. IK je bil izračunan s pomočjo naslednje formul:

IK: masa mokrega tkiva (g)×100/masa školjke (g)

Za SOS test je bilo odvzetih 30 školjk. S SOS testom se testira preživetje školjk po izpostavljenosti na zraku brez vode. Na krožnik je bil postavljen filter papir in nanj školjke. Krožnik je bil vstavljen v mokri laboratorij. Preživetje školjk je bilo opazovano dnevno.

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