The impact of Mediterranean mussels (*Mytilus galloprovincialis*, LMK.) on the amount of microplastic in the sea

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Abstract:

The Mediterranean mussel (*Mytilus galloprovincialis,* Lamarck,) is an edible sea mollusk, with dark blue shells, 5 to 8 cm long, although it can reach up to 15 cm in length. It feeds on plankton and organic matter by filtering seawater. An average mussel filters up to 6 liters of water per hour. Plastic is the name for a wide group of synthetic and semi-synthetic polymers with different characteristics, usually obtained from fossil resources (coal, crude oil, natural gas), and often obtained from organic products (cellulose, sugar beet, corn, seaweed, etc.). Microplastic (MP) is any synthetic solid particle or polymer matrix, of regular or irregular shape and size ranging from 1 μm to 5 mm, of primary or secondary manufacturing origin, which is insoluble in water. Previous research also demonstrates that microplastic bioaccumulation occurs within each trophic level, leading to biomagnification through the food chain. The research aim is to determine whether Mediterranean mussels reduce the amount of microplastics from the sea and can they serve as biopurifiers of microplastics in the areas of mariculture. Measurement data are microplastics' amount, shape, color, and size. Samples of 0.5L were filtered and microscoped at a magnification of 400 x. The Microplastic Monitoring Protocol Trial suggested by Sutti et.al. (2020) was followed. The area with the highest amount of microplastic is the area without mussels. While there were no significant differences between fiber sizes from different locations. On the other hand, natural mussels' habitats have the largest MP fragments, while locations without mussels have the smallest fragments of MP, and the difference in the size of the MP fragments is statistically confirmed. Most of the samples contain fragments, and the most common color of all pieces of microplastic is dark blue.

Keywords: plastic, Adriatic sea, mussels` farms,

Research Question and Hypothesis:

1. Does the presence of mussels impact the amount of microplastics found in the sea?
2. Which sampling site will have the biggest variety in microplastic shapes, colors, and sizes?
3. What are the most common types of microplastic found in the sampling sites?

The hypothesis is that there will be a smaller amount of microplastics in sea samples from areas densely inhabited by mussels than in sea samples where there are no mussels, because of the properties of the mussels to ingest microplastics into their organism through filtration, and the fact that it accumulates in them (Miller et al., 2020).

Introduction and Review of Literature:

The Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) is an edible marine mollusk, with dark blue shells, pointed at the front and widened and rounded at the back, usually 5 to 8 cm long, although it can reach up to 15 cm in length (Bonham, 2017). It feeds on plankton and organic matter by filtering sea water. An average mussel filters up to 6 liters of water per hour. It is grown in pergolas. Pergolas are mesh nets with a diameter of 2-3 cm, usually 3 meters long. (Šarlija, 2021). Plastic is the name for a wide group of synthetic and semi-synthetic polymers with different characteristics, usually obtained from fossil resources (coal, crude oil, natural gas), and from organic products (cellulose, sugar beet, corn, seaweed, etc.). Polymers are long molecules composed of repeating molecular units. They can have different structures: linear, branched, networked, etc. (Sutti et al., 2020). The longevity and resistance of plastic are also the biggest reason for environmental pollution both on land and in the seas (Sivan, 2011). The first notes on the presence of plastic in the seas dating back to the 1960s, when the first examples of marine mammals becoming entangled in discarded nets and feeding seabirds with plastic were observed in the northern part of the Pacific Ocean. Digestion of polystyrene pellets was recorded in 1972 in 14 fish species on the east coast of North America (Ryan, 2015). Significant pollution of marine systems has been recorded in closed bays and bays that are usually in the immediate vicinity of populated areas. Research in the marine ecosystem revealed that chemical additives, plasticizers, agents that reduce flammability, antimicrobial particles, and titanium dioxide nanoparticles (TiO_2-NPs), which are added to improve plastic`s properties, represent dangerous environmental pollutants because they can bind pesticides, herbicides, heavy metals, and various other particles to the plastic surface. Microplastic is any synthetic solid particle or polymer matrix, of regular or irregular shape and size ranging from 1 µm to 5 mm, of primary or secondary manufacturing origin, which is insoluble in water (Frias and Nash, 2019).

Polymers with a higher density than seawater generally sink to the bottom (PVC), while polymers with a lower density remain on the surface or in the water column (PE, PP). Fouling of debris, further fragmentation, and release of additives into the sea affect their buoyancy as well as the position of microplastics in the water column (Lusher, 2017). When plastic is fragmented into microplastics, it is easily ingested by organisms such as mussels that are commonly consumed by humans (A. Khoironi et al., 2018). The result of the analysis of microscopic microstructures carried out by A. Khoironi et al. (2018) showed that mussels from the marine environment are contaminated with microplastics. Previous research also demonstrates that microplastic bioaccumulation occurs within each trophic level, leading to biomagnification through the food chain (Miller et al., 2020). Intake of microplastics has been confirmed in wild populations of numerous marine organisms collected from their natural habitats. In all biological systems, exposure to microplastics can cause toxicity, oxidative stress, and inflammatory injuries (Prata et al., 2020). The negative effect of microplastics on organisms is also manifested in the disruption of gene expression, enzyme activity, or oxidation of free radicals at the molecular level (Shah et al., 2017). In sea orgasms, these negative effects cause reduced fertility and metabolic disorders (Guzzetti et al., 2018). The inability of the human immune system to remove synthetic particles can lead to chronic inflammation and increase the risk of tumors (Prata et al., 2020). Some evidence suggests that the intestinal uptake of plastic particles is relatively low and largely depends on particle size. However, other evidence highlights that microplastic fragments disrupt key molecular signaling pathways, alter the composition of the intestinal microbiota and may cause epigenetic changes, including transgenerational effects that may be involved in the emergence of many metabolic disorders. These results have significant implications for early life exposure to microplastics and metabolic changes and obesity in humans. Changes in the intestinal microbiota of animals caused by the action of microplastics can disrupt physiological homeostasis, which leads to diseases of other organs such as kidney disorders, circulatory system disorders, inflammation and tumors, and neurological disorders (Kannan and Vimalkumar, 2021). M.N. Woods et al. (2018) concluded that the blue mussel (*Mytilus edulis*) can serve as a sink for microplastics in the coastal waters of the Gulf of Maine, therefore the research aim is to determine whether Mediterranean mussels reduce the amount of microplastics from the sea and can they serve as biopurifiers of microplastics in the areas of mariculture. The hypothesis is that there will be a smaller amount of microplastics in sea samples from areas densely inhabited by mussels than in sea samples where there are no mussels, because of the properties of the mussels to ingest microplastics into their organism through filtration, and the fact that it accumulates in them (Miller et al., 2020). The confirmation of the hypothesis could stimulate new ideas about solving the problem of introducing too much non-degradable plastic human waste from the sea into cultivated marine organisms intended for food, and a new way of growing marine organisms where mussels grown around fish farms would serve as biofilters and enable the inflow of the sea with a smaller amount of microplastics.

Research Methods and Materials

Locations

The sea samples were collected in August 2022.The samples were from three different groups of locations: A) mussel farms, B) areas where there are no mussels, and C) mussels in nature, i.e., places where mussels grow without direct human influence. Locations, where there are no mussels, served as a control in the research. We went to the farms in agreement with the mentor and were accompanied by our parents.

A) Mussel farms

B) Locations without mussels

C) Natural mussels' habitats

Figure 1 Sampling sites on the coast of Croatia

Mussel farm

A1. Farm 1 has pergolas at a distance between 6 and 30 m from the shore, and samples were taken at a depth of 0.5 m and at 6 m from the shore. GPS location: 44° 15' 51.5304" N, 15° 31' 47.4996" E.

A2. Farm 2 has pergolas at a distance between 300 and 500 m from the shore, and samples were taken at a depth of 0.5 m and at 300 m from shore. GPS location: 44°16' 2.82" N, 15°31' 35.6376" E.

A3. Farm 3 has pergolas at a distance between 50 and 150 m from the shore, and samples were taken at a depth of 0.5 m and at 50 m from shore. GPS location 44° 16' 5.574" N, 15°31' 20.226" E.

Natural mussels' habitat

B1. The sample from location 1 was taken at 15 to 20 m from the shore and at a depth of 0.5 m. GPS location: 44° 14' 10.4424" N, 15° 31' 21.9504" E.

B2. The sample from location 2 was taken at 15 to 20 m from the shore and at a depth of 0.5 m. GPS location: 44° 12' 17.7768" N, 15° 34' 34.2156" E.

B3. The sample from location 3 was taken at 15 to 20 m from the shore and at a depth of 0.5 m. GPS location: 44° 12' 29.2932" N, 15° 34' 5.1528" E.

Mussel-free habitat

C1. The sample from location 1 was taken at 100 m from the coast and at a depth of 0.5. GPS location: 44° 33' 80.18" N, 15° 04' 61.40" E.

C2. The sample from location 2 was taken at 100m from the coast and at a depth of 0.5m. GPS location: 44° 34' 71.63" N, 15° 06' 09.11" E.

C3. The sample from location 3 was taken at 120 m from the coast and at a depth of 0.5 m. GPS location: 44° 33' 15.53" N, 15° 07' 63.43" E.

Sampling

Samples were taken around pergolas in mussel farms, and in natural mussels' habitats and habitats without mussels, At the mussel farm, the sample was taken among the pergolas. The nearest locations were reached by swimming and more distant by boat. At each location, three 500 mL bottles were filled at a depth of 0.5 m. The samples were taken by immersing the bottle under the sea and closing it under the sea. The samples were then labeled with the name of the location where the sample was taken, as well as general information about the location (depth, distance from the coast, sea temperature, pH, and sampling date. Taking into consideration that pH balance and water temperature affect the amount of microplastics, the GLOBE hydrology protocols for water pH and water temperature were used as a control factor to make sure they did not affect the results. A total of 27 seawater samples were collected. After sampling, the bottles were stored in a portable refrigerator and were frozen and transported to the school. Daily processing and analysis of samples were done in the school laboratory. The equipment used consists of; pH meter, thermometer, vacuum filtration funnel, and a vacuum pump, drawn filter membranes with a diameter of 47 mm and a pore width of 0.45 μm, a Petri dish with a diameter of 55 mm, tweezers, a sterile syringe, ionized water, a marker, latex gloves, a white corner, a table lamp, and a microscope with a camera (model DN-107T), laptop and USB cable. The samples were filtered using a vacuum pump and a funnel for vacuum filtration. During filtration, special attention was paid to the possible contamination of the filter and equipment, therefore, between the filtration of each sample, the filtration apparatus was cleaned with ionized water. To prevent contamination, several measures were implemented, including cleaning the table with 70% alcohol, using a white laboratory coat, tying hair, not wearing makeup and nail polish, and using transparent latex gloves. Seawater from 500 ml bottles was transferred to a funnel with a membrane filter for vacuum filtration, which was connected to a vacuum pump, and after filtration, particles larger than the membrane pore width (0.45 μm) remained on the membrane itself. When the sample was filtered, 10 ml of the ionized water was added and the filtration was completed, the membrane was moved and sealed in a Petri dish that was marked with a coded location tag.

Figure 3 Vacuum filtration equipment

Figure 2 pH meter and termometar

Dry filtrate membranes were analyzed using a school microscope model DN-107T at magnification 400x. Before the analysis of microplastic samples, the protocol was studied (Sutti et al., 2021; Sutti 2020), and microplastic recognition training was done with a mentor using a database of several hundred photographs. The size of microplastics is one of the elements of analysis, therefore calibration was made with a measuring scale inside the eyepiece of the Olympus CX23 microscope, where the distance between two lines at 100x magnification was 10 µm and the thickness of the filter paper line was determined to be 22 µm. Additional calibration was done with the help of the ScopeImage 9.0 program, which was calibrated by an automatic function in the system and confirmed the same thickness of the filter paper line. Samples were analyzed for microplastic fragments and plastic-origin microfibers, including their diameter, color, and shape. Each particle was photographed. The number of microplastic particles in a 500 ml sea sample was determined by counting all identified microplastic particles on the filtrate membrane. The collected data were entered in a table for all 27 samples.

Results:

Table 1 Temperatures and pH levels of sampling sites

	Mussel farms			Natural mussels' habitats			Locations without mussels		
Locations	Brkljača	Valmar	Dagnja	Novigrad 1	Novigrad 2	Maslenica	Vir 1	Vir 2	Vir 3
pH	8.0	8.1	7.9	7.8	7.6	8.3	7.7	8.0	8.1
T /°C	21.5	22.6	22.2	20.5	21.4	21.2	23.8	22.8	23.6

The results of the work indicate that the average number of pieces of microplastic is the highest in areas where there are no mussels, which can be seen in Figure 4.

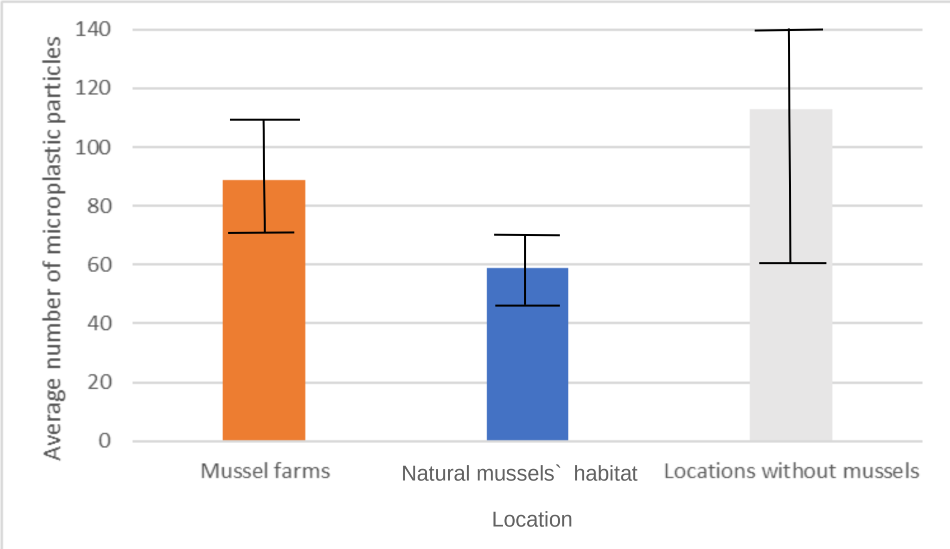

Figure 4 Comparison of the number of microplastic particles found in each location type

The average number of pieces of microplastic in mussel farms was 89 pieces; 85 fragments and 4 fibers were on average. For natural mussel habitats, the average number of pieces of microplastic is 61; 51 are in the form of fragments, and 3 in the form of fibers. Areas, where there are no mussels, have an average of 128 pieces of the microplastic present; 113 are in the form of fragments and 15 in the form of fibers.

The analysis of microplastic fragments size is shown in Figure 5. The fragments from each type of location are of similar proportions, but the largest spread of data around the average is shown by the wild mussel areas and the largest deviation by the mussel farm.

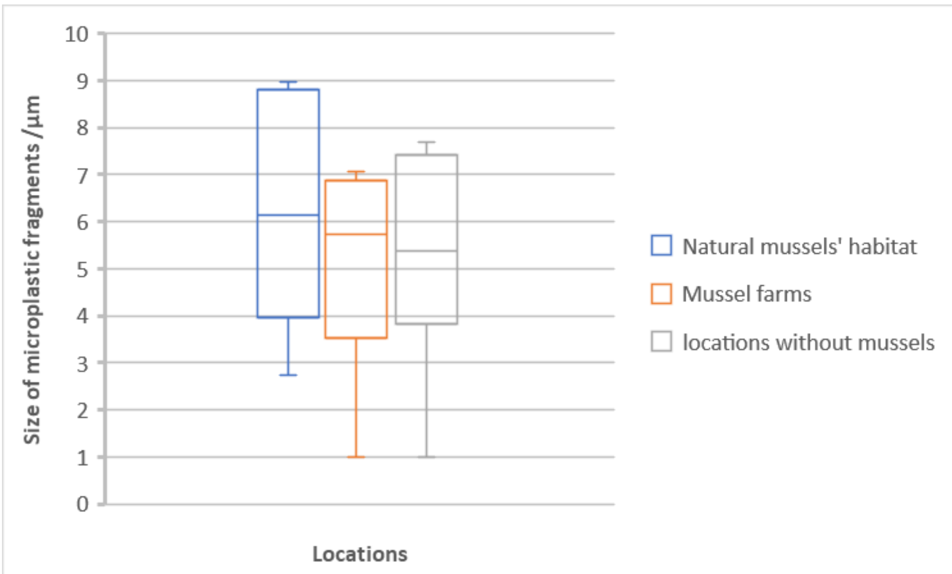

Figure 5 Microplastic fragments size comparation

The blue line in the middle of the box represents the average size of microplastic fragments found in the natural mussels' habitats. The boxplot shows how the data is distributed by taking 50% of the given data values. The blue vertical lines (whiskers) on the rectangle represent the maximum (above the rectangle) and minimum (below the rectangle) values of the size of fragments. The orange line in the middle of the box represents the average size of microplastic fragments found in the mussel farms. The boxplot shows how the distribution of data. The orange vertical lines (whiskers) on the rectangle represent the maximum (above the rectangle) and minimum (below the rectangle) values of the size of fragments. The grey line in the middle of the box represents the average size of microplastic fragments found in the locations without mussels. The boxplot shows how the distribution of data. The grey vertical lines (whiskers) on the rectangle represent the maximum (above the rectangle) and minimum (below the rectangle) values of the size of fragments.

Further analysis using the ANOVA test revealed a significant difference in the sizes of microplastic fragments ($F = 10.59$, $p < 0.0001$). The Tukey HSD post hoc test shows significant differences between the fragment sizes of all three locations, it was determined that $p < 0.0001$ for all.

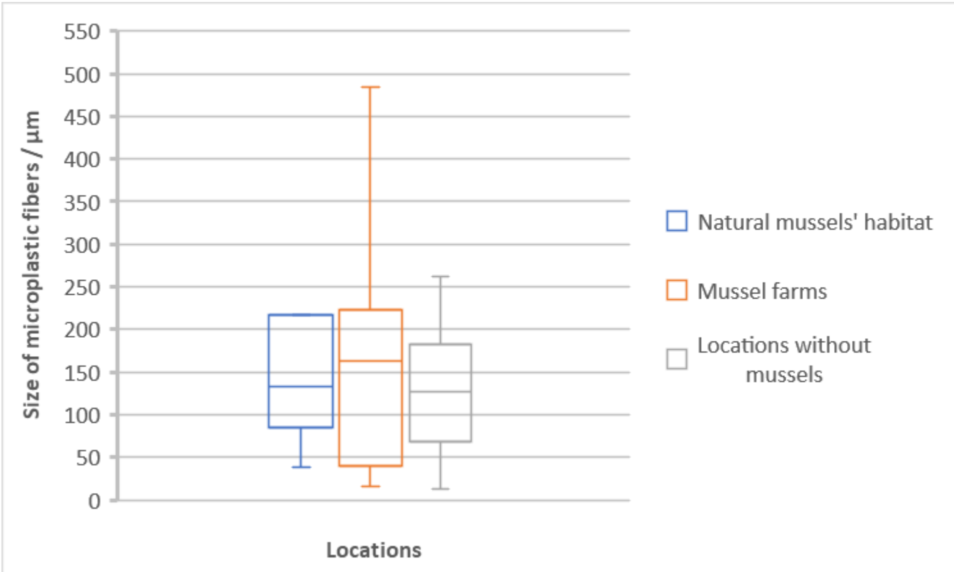

Figure 6 Microplastic fibers size comparation

The results in Figure 6 show that the mussel farms have on average the longest fibers and the highest fiber size dispersion, which indicates the diversity in the data. Only natural mussels' habitats show a value that deviates significantly from the fiber length average.
The blue line in the middle of the box represents the average size of microplastic fibers found in the natural mussels' habitats. The boxplot shows how the data is distributed by taking 50% of the given data values. The blue vertical lines (whiskers) on the rectangle represent the maximum (above the rectangle) and minimum (below the rectangle) values of the size of fibers. The orange line in the middle of the box represents the average size of microplastic fibers found in the mussel farms. The boxplot shows how the distribution of data. The orange vertical lines (whiskers) on the rectangle represent the maximum (above the rectangle) and minimum (below the rectangle) values of the size of fibers. The grey line in the middle of the box represents the average size of microplastic fibers found in the locations without mussels. The boxplot shows how the distribution of data. The grey vertical lines (whiskers) on the rectangle represent the maximum (above the rectangle) and minimum (below the rectangle) values of the size of fibers.

Further analysis using the ANOVA test determined that the difference in the sizes of microplastic fibers is not significant. ($F = 1.46$, $p > 0.0001$). Tukey HSD post hoc shows that there is no significant difference between any of the investigated locations.

Discussion:

The largest amount of microplastics were found in locations where there are no mussels, thus confirming the hypothesis that there will be fewer microplastics in areas with a high concentration of mussels than in areas where there are no mussels. Despite the higher concentration of mussels in mussel farms, the concentration of microplastics in farms is higher compared to mussel areas in nature. This is probably a consequence of the pollution of the location using plastic tools in cultivation. In the areas where there are no mussels, an average of 128 pieces of microplastic per m^2 were found. Schmidt et al. (2018) conducted research on the amount of microplastics in the Mediterranean Sea, and depending on the location, their average varies from 34 to 212 pieces per m2. These results coincide with the obtained results of this research. On the other hand, Glavičić Marović (2022) in her research uses the same method as this research, and in researching protected areas of the Adriatic Sea, she found about 10 pieces of microplastic per 500 mL of seawater but using lower power magnification (100x). Given that most research on microplastics investigates their chemical structure or bioaccumulation in marine organisms, it is difficult to compare the results with previous research. Of all the pieces of microplastic found, 86.5% were dark blue in color, and the greatest diversity in microplastic colors was observed in mussel farms. This is most likely also a consequence of pollution from plastic tools used during cultivation.

More reliable results could be achieved by observing the amount of microplastics in given locations over a longer period, and by more precise counting and measuring pieces of microplastics.

Conclusion:

There is indeed a lesser amount of microplastics found near the habitats of mussels, but regardless, human pollution of the sea is very visible and concerning. There is an undetermined source of dark blue plastic across the Croatian source that should be further investigated. The main form of microplastic in the sea is in a fragment shape, but there are also some fibers found. The largest fragments of microplastic are found in the natural habitats of mussels which could imply that there is less fragmentation happening. Mussel farms have the greatest deviations when it comes to the average size of microplastic fibers.

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Badge Descriptions/Justifications:

I make an impact.

While collecting samples along the Croatian coast I have met a great deal of mussel farmers, fish farmers and aquaculture workers. They all showed big interest in my research. Many of them would love to know how to make their business more environmentally friendly, especially small farmers who live in the area where they farm fish and/or shells. Cromaris is one of Croatia's biggest mariculture companies and they went out of their way to help me reach sampling sites that are usually only accessible to workers. By conducting this research, I was able to share the results with Cromaris and other smaller business owners, as well as ideas on how to downsize the amount of microplastics near their farms, which would then lead to raising the quality of their produce. Helping one farm at a time to adapt their ways of farming I was able to have a small impact on several people consuming their products.

I am a data scientist.

During this research I learned a lot about data sampling, analysis, and comparison.
I learnt that every good research should have a minimum of 3 samples so that statistical test could verify the significance of the results. So, to implement that rule every type of location I sampled had 3 sublocations, for example I collected data in 3 mussel farms and each mussel farm had 3 sea samples. Next in line of work was data preparation, which visualized itself in the form of vacuum filtration of collected samples. Then followed data processing or microscoping of all the filtered samples and recording the obtained data in Excel. Of course, I had to verify the significance of my data, which I did using ANOVA and Tukey HSD post hoc tests. Which then lead me to my own conclusions. After that I researched similar topics and compared my data to theirs. Only after all of that I could confirm the credibility of my conclusions.

I am a storyteller.

During my research I was mesmerized by beauty of Croatian coast, so I started taking photos of sampling sites, it quickly turned into documenting the process of research. I decided to share some of that process on my Instagram page, where I have a special memory reserved just for my microplastic journey. Some of the other photos include microplastic fibers and fragments in interesting shapes and the process of microscoping.