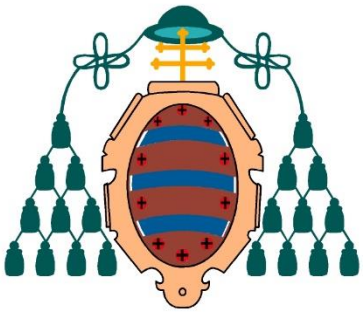

UNIVERSIDAD DE OVIEDO

MASTER UNIVERSITARIO EN CONSERVACIÓN MARINA

Master of Science in Marine Conservation

DNA Effects in *Artemia Salina* as a Model Organism Under Microplastics Exposure

TRABAJO FIN DE MÁSTER

Master Thesis

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Index

Abstract

The production of plastic started almost a century ago. Today, microplastic pollution is ubiquitous, we can find microplastics literally everywhere and the marine environment is not an exception. In the present study, we evaluate the effect of this pollutant on mortality and DNA degradation in the marine zooplankton species *Artemia salina*. Two experiments were carried out with two different population densities (low-density with n = 15 and high density with n = 100, in a total volume of 1,000 mL) and were exposed to three different concentrations of polystyrene microbeads (C0: Control, C1: 0.02 mg/L, C2: 0.2 mg/L and C3: 2 mg/L) for 7 days and at a constant temperature of 20ºC. The results showed an increase in the accumulated mortality of individuals subjected to the highest concentrations. These effects were shown to be dependent on the factors of population density and exposure time. In addition, it was shown that individuals subjected to medium and high concentrations had a higher mortality risk compared to the lower density and the control. DNA degradation levels could not be correlated with either microplastic concentration or exposure time.

1. Introduction

Fragmented plastics were first reported as a contaminant in marine ecosystems during the 1970s (Cole et al., 2011a). Only half a century after its first detection, the contribution of this pollutant material from land to marine ecosystems was recorded at between 4.4-12.7 million tons in 2010 and is increasing dramatically year after year (Jambeck et al., 2015). Microplastics (MPs) enter marine ecosystems mainly through human activities such as fishing, agriculture and aquaculture, tourism and other recreational activities, as well as through industrial and domestic wastewater (Guzzetti et al., 2018).

Currently, most of the studies published to date describe MPs with a size or diameter of less than 5 mm (McCormick et al., 2014; Ory et al., 2018). This is the size of the microbeads used in household products (Castañeda et al., 2014). Although sizes of less than 1 mm are also common. Recently, the range of size that the plastic fragment must have to be considered MP, and was delimited between 1µm and 5 mm, considering those smaller than this size as nanoplastics (NPs) (Castañeta et al., 2020). They can also be classified according to their origin; depending on whether they were manufactured as microplastics from their origin (primary microplastics) or were broken down after manufacture, i.e., they were produced by the degradation of larger plastic waste (secondary microplastics) (Cole et al., 2011a).

They can appear in the environment with many different shapes and sizes, such as fibers, beads, flakes, etc. The form in which they appear can vary enormously and depends to a major extent on their function and original form, as well as on the degradation processes and types of erosion to which they have been subjected (Zhang et al., 2020).

Of the total primary MPs polluting water bodies, 98% come from land-based activities and only the remaining 2% from aquatic activities (Kershaw, 2019). Poor waste management practices, illegal activities, and daily accidental discharges such as those caused by the construction sector, industry, agriculture, domestic use, and tourism, are the main routes of entry of MPs into aquatic environments (Boucher & Friot, 2017). These

sources of contamination of aquatic systems are mainly human activity on the coasts, agriculture and livestock, and industrial production, being reported by several studies as the major contributors of microplastics to aquatic environments (J. Wang et al., 2017). MPs reach water bodies through rivers in the form of non-degraded materials such as bottles, packaging and various wastes derived from home and recreational activities (Cole et al., 2011a). Rainfall and runoff from sewage treatment plants are also important sources of MPs pollution (Auta et al., 2017; Da Costa et al., 2016; Nel et al., 2018). Several studies like (Cózar et al., 2014; Sagawa et al., 2018) reported a staggering 88% prevalence of MPs in the open water surface. This is partly due to its low density compared to saltwater.

The transport of PM depends on different characteristics such as density, which determines buoyancy, shape and size, which determine its behavior in currents, and on certain biological and chemical processes specific to each environment (Andrady, 2011). Evidently, those with higher densities tend to sediment. These have been reported to alter sediment properties. They increase permeability and thermal absorbance may affect the marine biota in the sediment (Cole et al., 2011b). These types of microplastics are usually composed of higher density materials or have a higher amount of fouling organisms such as microalgae. They can be found from the seabed, closer to the beaches to the ocean depths (Woodall et al., 2014; W. Zhang et al., 2017). On the other hand, there are the MPs composed of lighter materials, although their shape also plays a role. Some of these polymers are for example polystyrene, polypropylene and polyethylene. These types of low density polymers tend to be more present in the surface layers and in the water column (Derraik, 2002; Gregory, 2009).

Another factor to take into account is that due to the large surface/volume ratio of the MPs and their hydrophobic character, they are perfect vectors for some pollutants, such as aromatic hydrocarbons (Wessel et al., 2010) heavy metals and polychlorinated biphenyls (PCBs) (Koelmans, 2015), among others. In addition, the additives that are incorporated during their production (phthalates, flame retardants, UV stabilizers, solvents, surfactants, …) can accumulate in the tissues of organisms that ingest them and travel along the entire food chain (Koelmans, 2015; Setälä et al., 2014).

The ubiquity of MP pollution in the marine environment has been reported for a long time (Barnes et al., 2009; Thompson et al., 2004), were some of the first to report it. At present, the situation is no better and it is known that the bodies of water near population centers are where the largest quantities are found (Ashrafy et al., 2023). Along with this wide distribution, its durability and diversity of sizes, makes possible its interaction with a large number of organisms belonging to different taxa and at all trophic levels is widely reported (Baulch & Perry, 2014; Duncan et al., 2017; Laist, 1997; Lavers et al., 2014). Especially the smallest MPs, since they are highly bioavailable for ingestion, and if, in addition, their size is equal to that of the potential prey, the bioavailability is even higher (Galloway et al., 2017). There are various adverse effects reported, from physical damage (Gall & Thompson, 2015), reduced feeding (Cole et al., 2015), growth, development or reproduction (Lo & Chan, 2018), oxidative stress (Jeyavani et al., 2022), genotoxicity (Albarano et al., 2022), gene expression (Choi et al., 2020) to DNA damage (Masiá et al., 2021; Pannetier et al., 2020).

Continental shelves, due to their high biological productivity and their proximity to areas of terrestrial pollution, are the places where there is the greatest risk of ingestion of MPs (Clark et al., 2016). Organisms such as zooplankton are abundant in these areas and due to their feeding behavior in surface waters, they are among the most at risk of ingestion (Cózar et al., 2014). Zooplankton is one of the main food sources for secondary producers and is therefore the entry route for these contaminants into the food chain (Setälä et al., 2014). There are several studies that relate the intake of MPs to their transfer between trophic levels (Farrell & Nelson, 2013; Nelms et al., 2018; Setälä et al., 2014; Watts et al., 2014). In the study carried out by Setälä et al. (2014), the transfer of 10 µm diameter polystyrene microbeads through mesoplanktonic to macroplanktonic species was demonstrated for the first time.

Given the importance and abundance of MPs, there are many studies that have focused not only on quantifying the presence of these emerging pollutants, but also on the harmful effects that their ingestion can produce, both on zooplankton and on many other species. For example, Masiá et al., (2021) observed worse physical condition and DNA damage upon exposure to different concentrations of virgin polystyrene microspheres in the Mediterranean mussel, *Mytilus galloprovincialis*, with sublethal effects. In the same way, Bergami et al., (2017) evaluated the effects of 40 nm diameter (nanoplastics) on microalgae and the brine shrimp *Artemia franciscana*, and Albano et al. (2021), evaluated how the ingestion of 10 µm diameter polystyrene microspheres in *Artemia salina* resulted in an effect on their feeding behavior and life cycle, however, but without finding lethal effects (Albano et al., 2021). These last two examples, working on the *Artemia* genus, are of added interest, since they are part of zooplankton, being an input of MPs by ingestion from the most basal levels of the trophic chain.

Subsequently, other studies have focused on the harmful effects of MPs ingestion both in zooplankton and in many other species. (Bergami et al., 2017) evaluated the effects of 40 nm diameter NPs on microalgae and the brine shrimp *Artemia franciscana.* (Albano et al., 2021) evaluated as the ingestion of 10 µm diameter polystyrene microbeads in *Artemia salina,* resulted in an effect on feeding behavior and life cycle, however no lethal effects were found.

The objective of this study is to investigate whether exposure to three different concentrations of polystyrene microbeads has effects on *Artemia salina* as zooplankton species model. The mortality and effects on DNA integrity of the zooplankton will be analyzed. Also, we will check whether certain parameters such as time and density of the populations of exposed specimens have significant effects on the results. For this purpose, two experiments with two population densities of *A. salina* adult individuals (n = 15 and n = 100) were carried out. In these experiments, the individuals were exposed to different concentrations of virgin polystyrene microbeads for a short period of time (7 days). During this time, the specimens were kept under fasting conditions, no additional substances were added to the microbeads and mortality was recorded every 24 hours.

2. Material and methods

2.1. *Artemia salina*

Artemia salina (Linnaeus, 1758) is a primitive arthropod that normally inhabits hypersaline water bodies and belongs to the *Artemiidae* family, which is approximately 100 million years old. Its body consists of three segments: head, thorax, and abdomen. Adult individuals vary in length from 8-12 mm, depending on the genus. In their natural habitat they feed on algae, protozoa, and detritus, having an active feeding behavior of non-selective filter feeders with a suspended particle size of 40-60 mm. They can survive in extreme conditions of both salinity and oxygen. (Gajardo et al., 2002).

Since they are used as live food for aquariology, they are supplied regularly and reliably. These characteristics and its ease of breeding, make the commonly known as "brine shrimp" an ideal model for experimentation (Rajabi et al., 2015).

2.2. Experimental design

The experiment was carried out at the facilities of the Aquarium of Gijón (Asturias, Spain). Eight bottles were filled with one liter of seawater from the Gijon Aquarium supply. This water passes through a 20 µm pore mesh and chemical filters at a depth of approximately 8 m, and several hundred meters away from the coast. The filled bottles were left inside the container with water at a constant temperature of 20ºC for tempering.

Individuals of *A. salina* supplied by the aquarium food company "Aquátikos", were randomly selected and they were kept for 2 hours in a container with oxygenation and warm water supply for acclimatization. After that, they were deprived of food for a period of not less than 12 hours to purge the digestive tract as far as possible. From these preselected *Artemias*, two experiments were carried out under the same MPs conditions, one with a low density of Artemia (n=15) other with a high density (n=100). Individuals were added with a Pasteur pipette and must be kept in a minimum volume of water. Once the *Artemias* has been transferred to a 50 ml beaker, it is leveled and poured into the bottle where the corresponding treatment will be carried out. Thus, a final volume of exactly 1,000 mL is achieved.

The microplastic solutions corresponding to each treatment were added in six different bottles, two per each treatment: C1= 0.02 mg/L, C2=0.2 mg/L, and C3=2 mg/L. These concentrations were selected from previous studies (Masiá et al., 2021). C1 represents realistic levels of MPs, similar to those we can find in the environment (Paul-Pont et al., 2018), and higher doses ,C2 and C3, were selected in accordance with experiments performed by Lu et al. (2018) in zebrafish (*Danio rerio*), and Wang et al. (2021) in mussels (*Mitylus curuscus*). Two extra bottles without MPs were used as control.

During the whole experiment the specimens were kept without food. After every 24h a mortality count was performed. Mortalities were stored in 1.5 mL tubes with 96% ethanol. These tubes were correspondingly labeled by treatment and day. After this, microscopic observations were made to check the contents of both the external appearance of the specimen and the contents of the digestive tract. After the first 24 hours of storage, the

ethanol was renewed from each tube and stored for an indefinite period of time in a refrigerator.

2.3. Microbeads employed

Polystyrene microbeads were selected because of their density (1.05g/cm^3), which gives them virtually neutral buoyancy, and they can be in the water column as well as on the seabed. Polystyrene has already been documented to release chemicals used during its manufacture that can potentially cause harmful effects in marine animals (Browne et al., 2007). Additionally, their small diameter of 10 µm (std dev < 0.2 µm, Coeff. var < 2%) allows them to translocate into the circulatory system as demonstrated in previous experiments with mussels (*Mytilus edulis)* (Browne et al., 2008).

The polystyrene $(C_8H_8)_n$ microbeads employed in this experiment are negative charge-stabilized colloidal particles. The microbeads are produced by polymerization of styrene under conditions that induce spontaneous coalescent bead formation. The polystyrene microbeads are supplied as aqueous suspensions (10% solids) composed mainly of polymer particles and water, with small amounts of surfactant, sodium bicarbonate and potassium sulfate.

A polystyrene bead contains water (> 69.0%), polymer (30.0%), surfactant (0.1-0.5%), inorganic salts (0.2%) (Sigma Aldrich, Germany, ref: 72986-5ML-F).

2.4. DNA Extraction and electrophoresis

First of all, the samples were removed from the refrigerator and kept at room temperature (15-25°C) for a period of time for tempering. Samples were pooled according to the number of individuals so that there was at least 25 mg of tissue for each extraction. Extractions were performed following the DNeasy® Blood & Tissue Kit protocol for tissues. In abbreviated form, samples were placed in 1.5 mL microcentrifuge tubes. 180 µL of ATL Buffer and then 20 µL of Proteinase K were added and then mixed by vortexing and incubate at 56ºC until completely lysed. The total lysis process took about 1 hour. However, the calcareous skeletons of the Artemia did not dissolve and had to be removed with a micropipette. After this, DNA purification was carried out by successive centrifugations and adding 96% ethyl alcohol and different buffers (AL, AW1, AW2). Finally, Buffer AE was added and centrifuged again. This last step was repeated to increase DNA yield, obtaining a final volume of 250 µL.

Groups of different numbers of individuals were made, so that there was a sufficient amount of tissue to correctly visualize the $_G$DNA bands on the corresponding agarose gel. The extracted DNA was quantified with Qubit-4-fluoromete (Qubit © 2021 Thermo Fisher Scientific Inc.). After this, calculations were made to adjust the volumes to have the same amount of DNA in each case, and to be able to run an agarose gel.

A 1.3% TBE 1x 50 ml TBE + 0.65g agarose + 2.5 µl simple safe agarose gel was then run with the corresponding µL of sample and the proportional µL of bromophenol blue sucrose solution and loaded into each well. Finally, 14 µL of DNA Molecular weight marker Perfect™ 100–1000 bp (EURx) was employed as ladder. Then, the DNA was

visualized under UV illumination NuGenius (Syngene) and photographed with a camera integrated.

The DNA integrity was inferred from the migration pattern in the gel. Samples were classified into four different categories, depending on the level of DNA degradation, following criteria based on (Quinet et al., 2016). Group 1: the DNA does not present any damage; the genomic band is perfectly defined and compact. Group 2: when the genomic band is blurred, and the smear is clear, it is considered a slight DNA damage. Group 3: When the genomic band is difficult to see, and the smear is more conspicuous and distinguishable, the DNA is considered to be quite degraded. Group 4: when the genomic DNA is non-existent and there is a large amount of smearing, the DNA is considered to be totally degraded. Once the corresponding gels have been developed, three independent observers must assign each sample to a specific group. Finally, averages are performed and a definitive group is assigned to each sample.

2.5. Data Analysis

All statistical analyses were carried out using RStudio software (R Core Team, 2022). The "dplyr" package (Wickham, François, et al., 2023) was used to manipulate the raw data grammar, the "tidyr" package (Wickham, Vaughan, et al., 2023) was used to sort the raw data names and the "ggplot2" package (Wickham, 2016) was used to plot the data in graphs. The mortality record was displayed for both the low-density experiment (where n = 15) and the high-density experiment (where n = 100) in two ways: First for residual mortality and then for accumulated mortality. Due to the small difference between the different treatments, an analysis of variance (ANOVA) was performed to determine if there were significant differences with respect to the variables: "time", "population density" and "treatment". Finally, to reinforce the results obtained, a Risk/Odds test (Risk Ratio) analysis was performed using as variables the accumulated dead and non-deeds, to analyze whether there are differences in mortality risk in two different groups. The first group consisted of the control and the diluted concentration (C0 and C1), and the second group consisted of the medium and saturated concentration (C2 and C3).

3. Results

3.1. Microscopic observations

The microscopic observation of the samples from both experiments showed MP in all cases, including the controls, due to the MP present in the water not retained by the filtration system Figure 4. As the concentration of MPs to which *artemias* are exposed increases, a higher density is observed within the individuals analyzed. Mainly, MPs tend to lodge in the gastrointestinal tract, with a maximum presence in the final portion. Larger amounts were found adhered to the remains of feces not yet excreted (Table 4).

3.2. Mortality

The highest mortality rates were observed after the first 24h from the start of both experiments, low and high density, after 48h of exposure, the mortality started to decrease for all MPs concentrations (Table 1).

a)

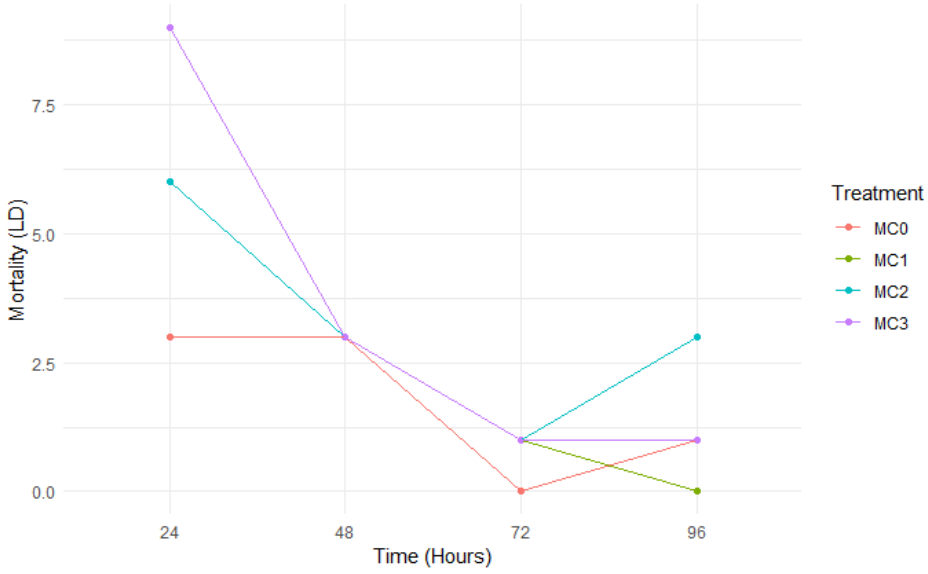
Time	MC0	MC1	MC2	MC3
24h	3	9	6	9
48h	3	3	3	3
72h	0	1	1	1
96h	1	0	3	1

b)

Time	MC0	MC1	MC2	MC3
24h	22	25	30	27
48h	20	20	30	33
72h	11	11	7	11
96h	10	1	6	2
120h	2	4	0	0
144h	0	3	1	4
168h	0	1	1	1

Table 1: a) residual mortality in low density experiment. b) residual mortality in low density experiment MC0: Control, MC1: c = 0.02 mg/L, MC2: c = 0.2 mg/L and MC3: c = 2 mg/L.

a)



b)

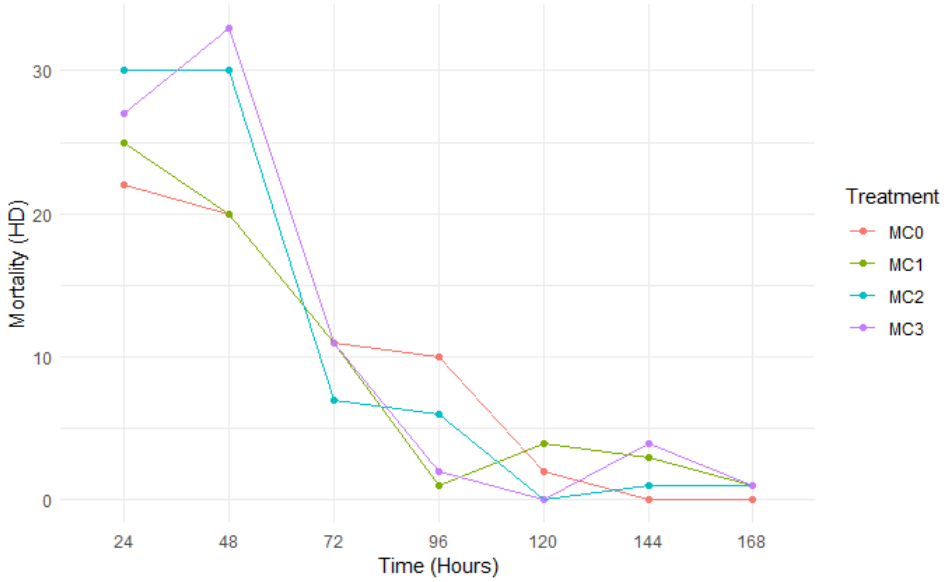


Figure 1:a) residual mortality in low density experiment. b) high density experiment MC0: Control, MC1: c = 0.02 mg/L, MC2: c = 0.2 mg/L and MC3: c = 2 mg/L.

3.3. Anova

ANOVA test results determined that the effects of the different concentrations were not significant over the mortality, respect to the control. However, population density and time of exposition were found to be a significant determinant of mortality. "density_type": -13.58442, (Pr(>|t|) = 1.14E-09 and "time": -0.17819, (Pr(>|t|) = 5.59E-12 (for density and time factors, respectively) (Table 2).

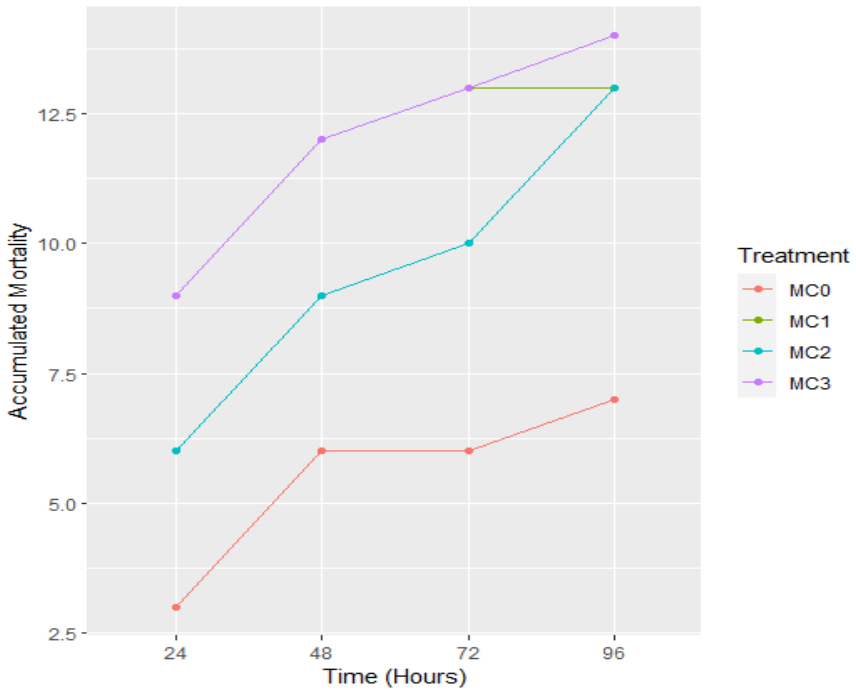
ANOVA HD and LD					
lm(formula = mortality ~ expr + density_type + time, data = mdf)					
Residuals:					
Min	1Q	Median	3Q	Max	
-8.9708	-2.8919	-0.1407	2.944	13.4762	
Coefficients:					
	Estimate	Std. Error	t value	Pr(>|t|)	
MC1	0.54545	2.1328	0.256	0.8	
MC2	1.45455	2.1328	0.682	0.499	
MC3	1.81818	2.1328	0.852	0.399	
density_type	-13.58442	1.69813	-8	1.14E-09	***
time	-0.17819	0.01814	-9.823	5.59E-12	***

*Table 2: Residual standard error: 5.002 on 38 degrees of freedom. Multiple R-squared: 0.7569, Adjusted R-squared: 0.7249. F-statistic: 23.66 on 5 and 38 DF, p-value: 9.87e-11. Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

3.4. Accumulated Mortality

Analysis of the accumulated mortality data from both experiments confirmed the significance of the effects of time and population density. It also showed slight but non-significant differences as the MP concentration increased (Figure 2).

a)



b)

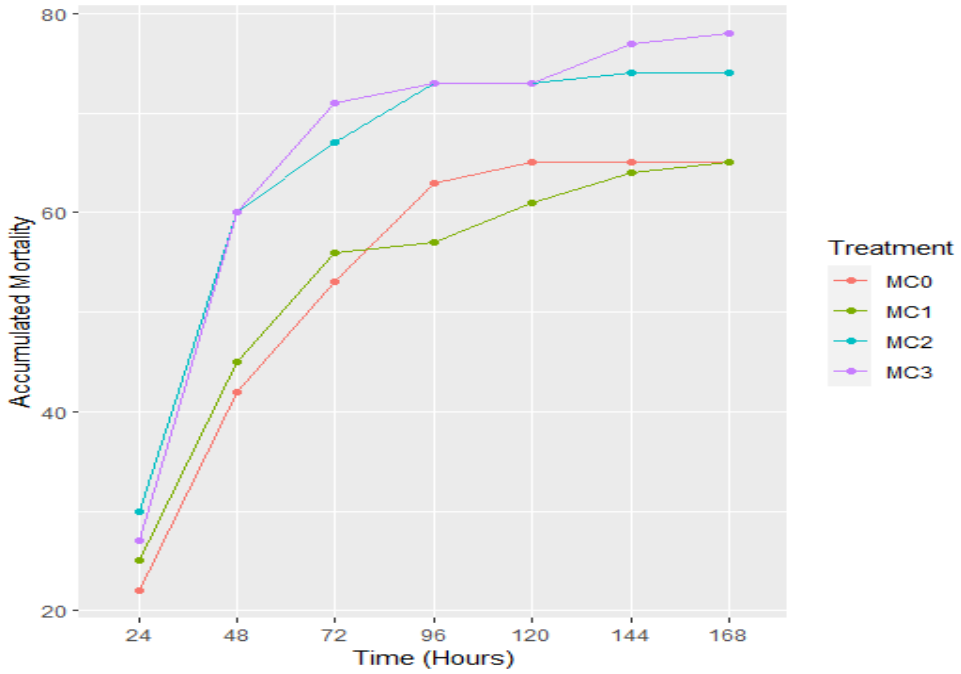


Figure 2: Accumulated mortality in low density experiment. b) high density experiment MC0: Control, MC1: c = 0.02 mg/L, MC2: c = 0.2 mg/L and MC3: c = 2 mg/L.

3.5. Risk/Odds test

In first place the results of the risk ratio (RR) test on the effect of the concentration of MPs on accumulated mortality determined a higher probability of mortality in group 2 (C2 and C3) than in group 1 (Control and C1) in both experiments. The results for the low-density are risk ratio = 0.55319 with a 95% confidence interval (0.3541-0.8642) where RR (z) = -2.6013 (p (ratio = 1) = 0.0092867. And for the high-density are risk ratio =0.76779 with 95% confidence interval (0.6318-0.9331) where RR (z) = -2.656 (p (ratio = 1) = 0.011894 (Table 3).

a)

Risk difference:	-0.3437
95% confidence:	[-0,6128 .. -0,07456]
z pooled:	-2.1936
p (same):	0.028266
z unpooled:	-2.5029
p (same):	0.012316
Risk ratio:	0.55319
95% confidence:	[0,3541 .. 0,8642]
z:	-2.6013
p (ratio=1):	0.0092867
Odds ratio:	0.22222
95% confidence:	[0,05404 .. 0,9138]
z:	-2.085
p (ratio=1):	0.037066

b)

Risk difference:	-0.13893
95% confidence:	[-0,2451 .. -0,0328]
z pooled:	-2.528
p (same):	0.011472
z unpooled:	-2.5658
p (same):	0.010294
Risk ratio:	0.76779
95% confidence:	[0,6318 .. 0,9331]
z:	-2.656
p (ratio=1):	0.0079075
Odds ratio:	0.57049
95% confidence:	[0,3684 .. 0,8835]
z:	-2.5153
p (ratio=1):	0.011894

Table 3: a) Risk/Odds test results of the low-density experiment. b) high-density experiment.

3.6. DNA integrity and electrophoresis

The agarose gel obtained after electrophoresis process does not show any correlation between the increasing concentration of MPs of the different treatments, neither population density, with the degree of DNA degradation. Nor is there any pattern that would indicate a direct correlation between the time of exposure to the treatments or the degree of DNA degradation. Genomic DNA (gDNA) is evident in all the wells, and some smear is present in some of them, however, it cannot be related to the different concentrations of MPs nor to the exposure time (Figure 3).

a)

b)

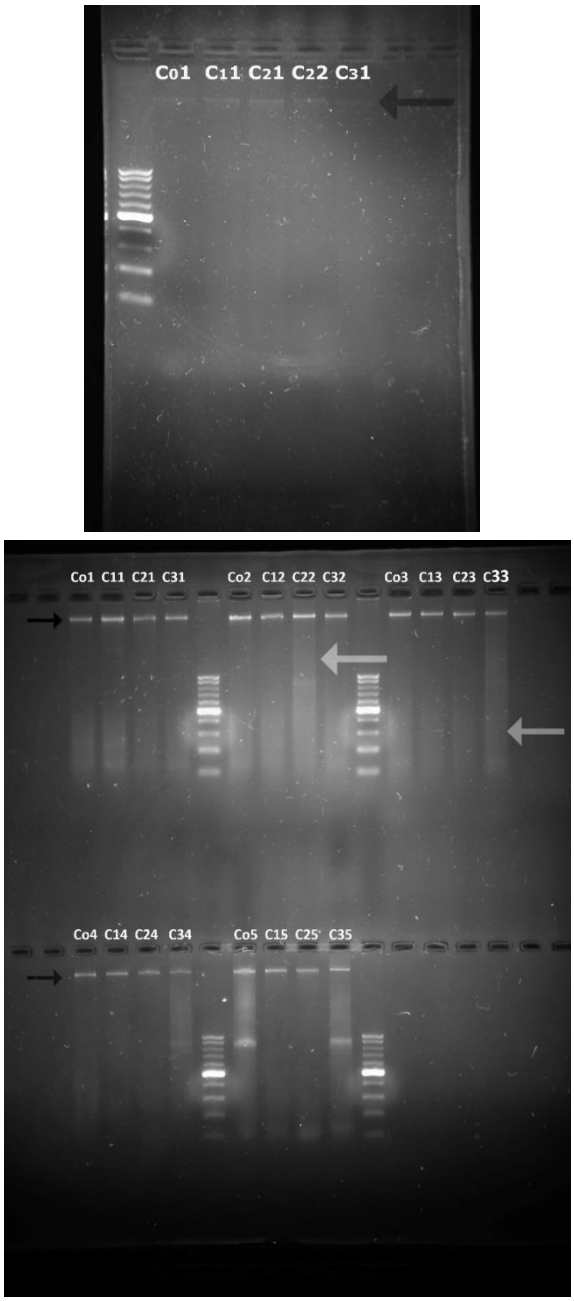


Figure 3: Agarose gel of the DNA extracted from individuals. a) 48.8 ng/µL in each well. C01= Control with 24-48h exposure, C11=C1 24-48h, C21=C2 t24-48h, C22=C2 t72-86h, C31=C3 t24-48h.
b) 80 ng/µL in each well. Cx1; t = 24h, Cx2; t = 48h, Cx3; t = 72h, Cx4; t = 96-144h, Cx5; t=168h. C0x = Control.

4. Discussion

The fact that microplastics are a ubiquitous pollutant in all water bodies means that they undoubtedly have an impact on organisms. The Mediterranean Sea is considered as a global accumulation zone of MPs with average concentrations of 243,854 plastics/m^2 of which 83% are MPs (Cózar et al., 2015). As for the Cantabrian Sea, the high rates of ingestion of MPs by fish found (Bellas et al., 2016) can be attributed to the oceanographic characteristics of this region (described in (Gago et al., 2015). The first observation of neustonic plastics in Spanish waters came from (Gago et al., 2015). In this work, they sampled along the NW coast of Spain during 2013 and 2014 with a trawl net fitted with a 333 mm mesh. Different types of plastics were found in 95% of the stations.

In 2016 a study was published documenting the ingestion of MPs by three of the most economically important demersal species; red dogfish (*Scyliorhinus canicula*), european hake (*Merluccius merluccius*) and red mullet (*Mullus barbatus*) in Spanish Atlantic and Mediterranean waters. The percentages of fish with MPs were close to 20% in all cases with a mean of 1.56 $\pm$0.5 elements per fish (Bellas et al., 2016). In addition, trophic transmission of MPs was demonstrated in laboratory conditions in 2022. This study was based on the analysis of prey (blue whiting *Micromesistius poutassou* and krill *Meganyctiphanes norvegica*) found in the stomach contents of european hake (*Merluccius merluccius*) (Cabanilles et al., 2022).

From our experiment, microscopic observations revealed the presence of microplastics in the digestive tract of all the individuals analyzed. From the first 24 hours, in all treatments, an increasing number of MPs can be observed according to the respective concentration in each case (Table 4). A striking, although not surprising, fact is that plastic particles from the medium were also found in individuals, including control samples (Figure 4). These particles are easily distinguishable from those used in the present study since they have different colors and shapes, as well as irregular sizes. It is impossible to know the origin and composition of these particles since they could come from the water supply of the Gijón Aquarium.

On the other hand, the present study has shown that the presence of polystyrene microbeads has a non-significant effect on *A. salina* mortality. The effects of different concentrations did not show significant differences in mortality. Similar results were also found in the experiments on chronic toxicity in *A. parthenogenetica,* with survival rates greater than 95 % regardless of treatment. Among all groups of *A. parthenogenetica* exposed to MPs (1 E1000 p/mL and 0.55 E550 mg/L) over a time period of 14 days ($p > 0.05$), no significant difference was found, including the control group (Wang et al., 2019). However, in the present study, population density and time of exposition were found to be a significant determinant of mortality, confirmed by the analysis of the accumulated mortality data (Figure 2). This slight tendency could be related to the accumulation of microplastics inside the body before affecting the mortality. This is shown in the study (Gambardella et al., 2017) where 48 hour exposures to concentrations of >1 mg/L to MPs (polystyrene microbeads) significantly altered the swimming ability of planktonic crustaceans (*A. franciscana* and *Amphibalanus amphitrite*). However, they contrast with the results reported by (Han et al., 2021) where a decrease in the survival and growth rate was demonstrated. The MP (polystyrene) and the concentrations used were the same as in this experiment (0.02 and 2 mg/L), but the effect of temperature was

also evaluated, showing in general, higher mortalities in treatments combining high temperatures and concentrations (T^{o}=30ºC and c = 2 mg/L), suggesting that different stress factor should be taken into account and could have cumulative effects.

It should be noted that during the daily removal of the dead and their subsequent observation under the microscope, the samples analyzed showed partial or total occlusions of their digestive tract. This could indicate abnormal feeding behavior. Individuals with a digestive tract full of MPs may have a reduced feeding capacity and this may affect their metabolism and life cycle. In fact, in the concentrated treatment (C3: c = 2 mg/L) at 144 hours, a nauplius with microbeads was found inside the intestinal cavity (Table 4). These observations are consistent with many other studies such as (Albano et al., 2021; Bergami et al., 2016; Botterell et al., 2019) corroborating how polystyrene microbeads significantly affected their food uptake (feeding) which was reduced by 50% in *A. salina* larvae (life cycle), and mobility (behavior).

If we compare the results of mortalities from both experiments (Table 1), we see a clear difference. In the low-density experiment (n = 15), low concentrations of MPs correlate perfectly with low mortality rates. However, in the high-density experiment (n = 100), this correlation is not so evident. This seems to indicate that population density plays a role. The explanation for this difference probably is the simplest, microplastics will be more available in a medium where, under the same conditions, there is a higher ratio of microplastics per individual. Many other studies have shown that higher abundances of MPs lead to higher ingestion rates and ultimately to the prevalence of adverse effects (Cole & Galloway, 2015; Kaposi et al., 2014; Messinetti et al., 2018).

The effect of population density was reaffirmed by the results of the analysis of variance that was carried out. Comparing the effect of the different treatments between the two experiments determined that population density had a significant effect on mortality. Additionally, we see that the time of exposure also played an important role in the increased mortality (Table 2). It is logical that the longer the exposure time to a pollutant, the greater the risk of being affected. Regarding the time of exposure, there are contradictory outcomes in the scientific literature, particularly when it comes to polystyrene particles. In the study carried out by (Cole et al., 2015) was assessed the toxicity of polystyrene microbeads in the marine copepod *Tigriopus japonicus*. In this study, mortality increased with time, suggesting that toxicity is a time-dependent factor. In contrast to this, (Gambardella et al., 2017) showed that different types of microplastics such as polystyrene did not affect mortality after 24-48 hour of time exposures.

This last argument reinforces the hypothesis of sublethal effects of polystyrene microbeads in *A. salina*. On the other hand, considering the results obtained for accumulated mortality, an upward trend can be seen as a function of the increasing concentration of MPs. Although the results of the analysis of variance showed non-significant values for the different treatments, a progression towards significance can be seen (Table 2). There seems to be a tendency towards higher accumulated mortalities in the more concentrated treatments (C2 and C3). The Risk/Odds tests showed a higher mortality risk in treatments with higher concentrations of MPs (Table 3). These results reinforce the hypothesis that the higher the concentration of MPs, the higher the accumulated mortality depending on the exposure time.

Regarding the results of DNA extractions and electrophoresis. Nor is there any pattern that would indicate a direct correlation between the time of exposure to the treatments or the degree of DNA degradation. Genomic DNA (gDNA) is evident in all the wells, and some smear is present in some of them, however, it cannot be related to the different concentrations of MPs nor to the exposure time (Figure 3). These results show that polystyrene microbeads do not produce a significant effect on *A. salina* DNA integrity in short periods of exposure (7days). This type of assay, although not new, is novel in planktonic crustacean species such as *A. salina*. Previously, different studies on the integrity of DNA exposed to MPs have been carried out, but always in other organisms.

One of the first studies that evaluated DNA integrity in marine organisms was in 2010 by (Wessel et al., 2010), however it did not use MPs but directly associated DNA damage in fish larvae (juveniles of *Solea solea*) with exposure to polycyclic aromatic hydrocarbons (PAH). In this study, exposure to Benzo[a]Pyrene (B(a)P), a type of PAH that, by ingestion causes increased DNA breaks in fish, was found to be the main agent of damage to genetic material in this case. In another experiment under laboratory conditions with larvae of the Japanese medaka (*Oryzias latipes*), they were exposed to a mixture of MPs (40% of low-density polyethylene, 25% of high-density polyethylene, 25% of polypropylene and 10% of polystyrene) coated with B(a)P showing significant induction of DNA strand breaks (Pannetier et al., 2020). Therefore, our results are consistent to the extent that we used virgin polystyrene microbeads without any additives. Few studies have been able to directly link the MPs exposure to DNA breakage; (Ficociello et al., 2021) demonstrated that degraded MPs exhibited a genotoxic effect on the nematode *Caenorhabditis elegans*, resulting in increased DNA degradation and apoptotic germ cells. Also, (Masiá et al., 2021) in his experiment with Mediterranean mussels (*Mytilus galloprovincialis*) found DNA damage after exposure to virgin polystyrene microbeads for a period of time of 21 days. These results contrast with those obtained in the present study, since the polystyrene microbeads used were not coated with additives, in fact they were the same. But in this case, it could be related to DNA degradation. However, there are some significant differences with the present study such as the longer exposure time, the high filtration rate of the mussels and the fact that the gills, which are directly in contact with the external environment, were the tissue analyzed.

The importance of this study lies in the fact that zooplankton species such as *Artemia salina* are often the routes of entry and transfer of MPs to higher levels of the trophic chain (Setälä et al., 2014). It is important to acquire knowledge about the translocation of MPs through the digestive tract to other organs (Cabanilles et al., 2022) in order to evaluate their possible transfer through the trophic chain (Lusher et al., 2017). Furthermore, it is difficult to establish clear correlations between different MPs studies, mainly due to the wide variety of different organisms and methodologies used for this purpose (Fang et al., 2019). The paradigm of the MPs has acquired such a relevance that nowadays it goes beyond the fields of ecosystem conservation. Today we are facing a problem that affects us directly and poses a serious hazard to the health of all the inhabitants of the planet, including its cause, the human being.

5. Conclusions

The concentration of MPs has not been shown to be a significant cause of mortality in *Artemia salina*. This poses an even greater problem in that higher survival rates mean greater bioavailability of MPs to subsequent steps in the trophic chain. However, factors such as time and population density have been shown to be of great importance in the accumulated mortality rates and risk of death of groups exposed to higher concentrations. The negative results in terms of DNA degradation should be taken with caution as future experiments should be approached at different temperatures, population densities and with longer exposure times (even over generations). Because of the great importance of research with MPs and their high disparity in terms of methodologies and model organisms used, it is necessary to unify criteria for a better understanding of the results and progress in the knowledge of the consequences.

6. Limitations and perspectives

From the beginning, approaching this project was very attractive both for its highly topical subject matter and its innovative approach. But, because of this reason, it was a difficult challenge to tackle. We had to face many problems such as not having previous experience in handling animals of such a small size, especially for DNA extractions. Trial and error became the method to follow. It was also not easy to find precedents in the scientific literature regarding DNA degradation assays using virgin MP-based treatments (without contaminant additives). In addition, due to the disparity of all previous studies in terms of methodologies and model species employed to evaluate the effects of MPs, it is difficult to compare and establish commonalities without a standardized methodology (Fang et al., 2019).

Due to the importance that the time factor has shown in the results. It would have been helpful to have had more time to carry out the project. Different studies have shown that MPs affect larval behavior, fecundity, and survival (Choi et al., 2020). This would imply doing long term experiments even during different generations.

7. References

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8. Annexes

Annex 1: Sampling Protocol for *Artemia salina.*

1. The aerator is removed from the bottle, dried on the outside with paper and left to rest on a table or white background. For a few minutes, the dead ones go to the bottom, where it is easier to catch them.
2. With the help of a headlamp and a modified Pasteur pipette (it is lengthened by inserting a thin pvc tube with a beveled end so as not to make a suction cup at the bottom). All the dead individuals are collected, deposited, and counted in a 50 ml glass beaker.
3. With a normal Pasteur pipette, they are transferred (in groups of 5 if possible) to an Eppendorf tube. With the same pipette and tilting the tube we remove as much water as possible taking care not to extract the artemia. It can also be done by introducing pieces of paper, but if we touch an individual, we will lose it as it will be totally adhered to the paper.
4. Once as much water as possible has been removed from the tubes, we proceed to add 96% ethyl alcohol. This first alcohol should be changed after 24 hours and then the one that can remain indefinitely should be added to preserve the samples.
   As alcohol is very permeable and Eppendorf tubes are not completely airtight, we must seal them with adhesive tape around the cap. In this way, they can be stored safely for an indeterminate period of time.
5. Label and store the samples in a safe place.
6. Clean all instruments thoroughly before reuse, taking care to properly remove all residues as they contain polystyrene microspheres.

Annex 2: Microscopic Observations

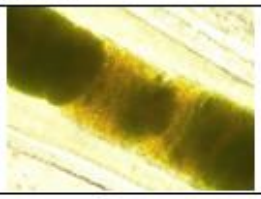
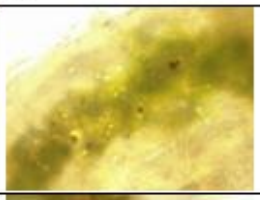
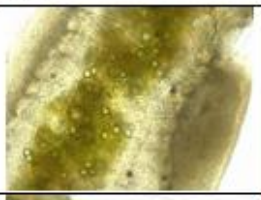
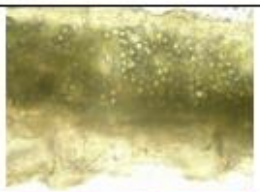
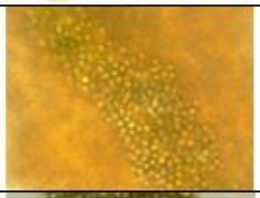
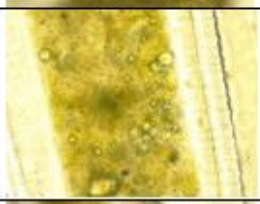
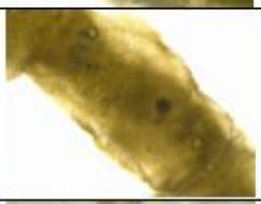
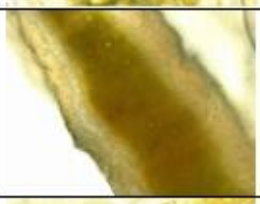
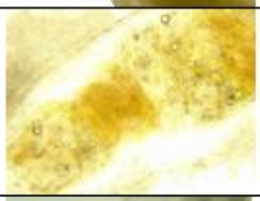
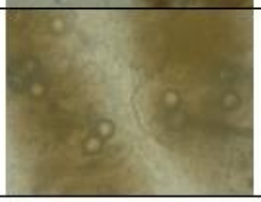
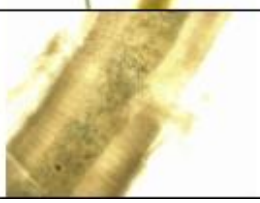
	Control	C1	C2	C3
24h				
48h				
72h				
96h				
120h				
144h				
168h				
Remaining				

Table 4: Microscopic Observations. Photographed with objective 4× and 8 x.

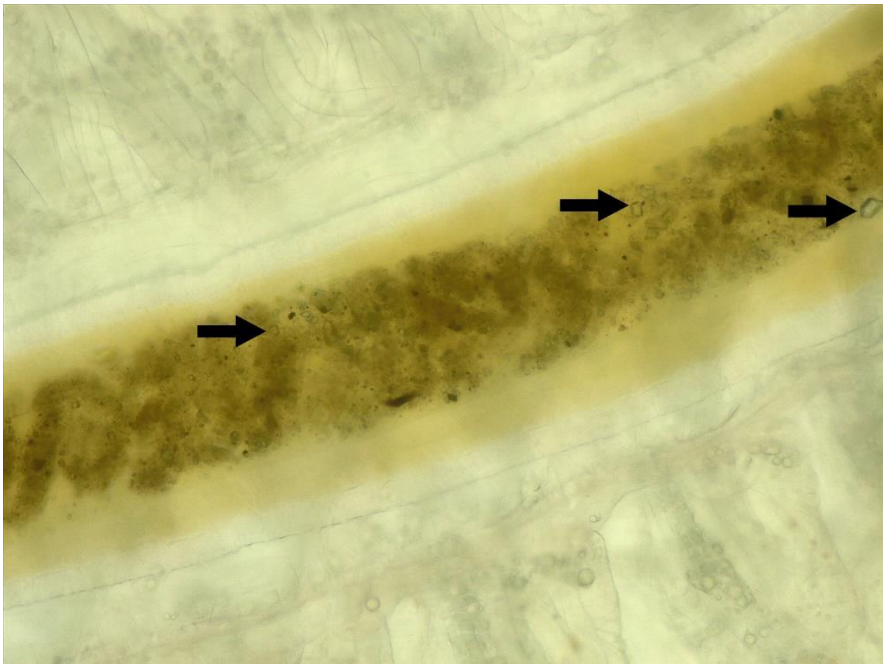


Figure 4: Control, 168h (Remaining) Microplastics of unknown origin. Photographed with objective 4×.