# NET4mPLASTIC PROJECT

## WP4 – Act. N 4.4 Microplastic and risks for Human Health

## D 4.4.3

Evaluation of clearance gut rate in bivalves for risk assessment associated with their consumption.

June, 2022 – Final version

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|---|---|
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# INDEX

# 1 Introduction

This document reports the details and related results of the experiment (conducted in triplicate) relating to the “Laboratory uptake and clearance” of mussels from microplastics conducted by IZSAM.

# 2 Materials and methods

## 2.1 Microplastics used for experimental tests

The microplastic particles used in the experiments were purchased on the market and/or supplied by PET recycling companies that have flakes or pellets as the final product.

| MPsType | Typepolimer | Sizeclass | Purchased |
|---|---|---|---|
| **Beads** | *Polystyrene RED* | - 100 µm<br>- 200 µm | ThermoScientific™ |
| **Fibers** | *Polypropylene* | - < 1000 µm<br>- 1000 -2000 µm<br>- 2000 -3000 µm<br>- > 3000 µm | LA MATASSINA |
| **Fragments** | *Polyethyleneterephthalate (PET)* | - 10 - 50 µm<br>- 50 – 100 µm<br>- 100 – 500 µm<br>- 500 -1000 µm<br>- > 1000 µm | Montello SPA |

## 2.2 Preparation of algal cultures to be used for feeding mussels during the experimental test

During the experimental tests it was necessary to feed mussels.
The bivalveswere fed with algal cultures of *Isochrysis galbana* and *Tetraselmis suecica* in a dose equal to 3% of their dry weight, as indicated by the authors Albentosa et al., 2012.
It was therefore necessary to start massive algal production equal to 150-200 liters per type of algae administered.
The times required for these productions are indicated below:

| Succession technique protocol | Time Range | Algal final density |
|---|---|---|
| From 50 ml flask to 250 ml flask | 7- 8 days | 3,2 x $10^6$cell/ml |
| From 250 ml flask to 2 liter flask | 5- 6 days | 1,9x $10^6$cell/ml |
| From 2 liter flask to 5 liter balloon | 5- 6 days | 3x $10^6$cell/ml |
| From 5 liter balloon to 150/200 liter big bag | 7- 8 days | 2x $10^6$cell/ml |

## 2.3 Experimental protocol "*Laboratory uptake and clearance*"

The experimental protocol included the following phases:

1. Acclimatization;
2. Exposure phase;
3. Purification phase.

### 2.3.1 Acclimatization

The mussels used for the experiments were taken from Defmar mussel farm located in Termoli. Each experimental group (one for each replica) was made up of 120 individuals.
Upon arrival at the laboratory, mussels were scrubbed to remove biofouling, then acclimated for 7 days by placing them in a glass aquarium system (50 L) containing filtered artificial seawater (Instant Ocean; 0.8 mm membrane filter, Supor® 800) aerated at 18 ± 1 °C and salinity 35 ‰.
During acclimatization mussels were under a photoperiod regime of 12 h light to 12 h dark, no food was supplied, the filtered artificial seawater was continuously aerated and changed

daily.This phase was necessary to allow them to clear their gut.

After 7 acclimatization days, 20 mussels (control group) was removed and prepared for the digestion and qualitative and quantitative analysis of the MPs, while the remaining organisms was prepared for a 3 day contamination phase.

### 2.3.2 Exposure phase

For the 3-day MPs exposure phase, the 3 main types of microplastics found in the marine ecosystem were used: granules, fragments and fibers.
For the contamination, a concentration of 2000 MPs/L of artificial sea water was used with a ratio of granules, fragments and fibers of 1: 1: 8, the same proportion found in marine environment according to the authors Su et al., 2016.
The experiment was performed at a constant salinity of 35‰, temperature of 18 °C, and a 12 h light-dark regime.
The mortality of mussels was monitored daily and the water was renewed to ensure that previously egested material, including microplastic particles, would not be ingested again.
Furthermore during whole period the mussels were daily fed with algal cultures of *Isochrysis galbana* and *Tetraselmis suecica* with a dose equal to 3% of the dry weight of the mussels, ration considered to be enough to fulfill the mussel's daily energy requirements (Albentosa et al., 2012).

### 2.3.3 Purification phase

At the end of the 3-day exposure period, mussels were removed from the exposure tanks and thoroughly rinsed to avoid any transfer of microplastics.
20 mussels (0-time group) were removed and prepared for digestion and qualitative-quantitative analysis of the MPs, while other organisms were moved to another tank with clean seawater and subsequently undergone to purification process.
The purification phase lasted a total of 7 days during which monitoring of mortality, renewal of water and feeding of the organisms were carried out on a daily basis.
At the end of the 2-day purification period, 20 mussels (2-time group) were removed and prepared for digestion and qualitative-quantitative analysis of MPs, while other organisms continued purification for up to 7 days.
At the end of the 7-day purification period, 20 mussels (7-time group) were removed and prepared for digestion and qualitative-quantitative analysis of MPs.

Two different purification times were applied:

- a "*microbiological*" purification lasting 2 days, corresponding to the maximum purification time applied for microbiological contaminants foreseen in shellfish purification plants;

- and "*experimental*" purification lasting 7 days, assuming to increase the dwell time in the shellfish purification plants up to 7 days if this time allows a better purification from these contaminants.

The same experiment in their entirety was repeated thrice.

## 2.4 MPs' qualitative and quantitative analysis

Digestion of mussel soft body was performed for each individual separately filling a glass bottle with 20 ml of 30% $H_2O_2$ for gram of soft tissues. The bottles were covered and placed in an incubator at 60-65 °C for 5-7 days.

Was adopted a blank extraction control without tissue but consisting of water and $H_2O_2$ in the same concentration used for the organic matter digestion, in order to correct the potential procedural contamination.

The obtained solution by each sample was filtered with glass microfiber filters, having a porosity of 2,7 µm (Whatman® glass microfiber filters, Grade GF/D, GE Healthcare, UK), using a vacuum pump.

The filters thus obtained were then be placed inside glass Petri dishes and left to dry at room temperature.

In the end, the filters were observed under a stereomicroscope (Leika MZ6, Leica Microsystem Ltd., Heerbrugg, Switzerland), images were taken using a digital camera (JVC-C1381, JVC, Yokohama, Japan) and the data acquired with software (Leica IM500 version 1.5, Leica Microsystem Ltd., Heerbrugg, Switzerland).

A visual assessment was applied to identify e classify types of microplastics according to the rules of Hidalgo-Ruz et al., 2012 and the Hot Needle Test based on De Witte et al., 2014.

## 2.5 Gene expression analysis

For each sampling time (control group, at the end of acclimatization phase; 0 time group, at the end of the exposure phase; 2 time group, at the end of the 2 days of purification; 7 time group, at the end of the 7 days of purification) five organisms were taken. From each bivalve separatly gills and digestive gland were taken and pools created, then have been submitted to bio-molecular investigations in order to evaluate a set of target genes, through the analysis of gene expression

with qPCR real-time to evaluate the response of bivalves to stress from microplastic pollutants.
Digestive gland and gills are both tissues of relevant interest for the analysis of the change in the expression of target genes. The first has been described as the organ in which pollutants accumulate in higher concentrations, while gills are the dominant site of interaction with the environment (Brandts et al., 2018).
The steps performed for the gene expression analysis are as follows:

1. RNA extraction;
2. cDNA synthesis;
3. Primers and PCR efficiency (*Eff*);
4. qPCRSybr Green.

### 2.5.1 RNA extraction

Digestive glands and gills for eachsampling time (control group, 0 time group, 2 time group and 7 time group) were dissected and quickly snap-frozen in RNA later™ Stabilization Solution and stored individually at 80 °C.
Digestive glands and gills were then pooled (4-5 individual per pool) according to sampling time and subjected to mechanical homogenization using quartz powder and pestle.
For each pool, total RNA was subsequently extracted using Quick-RNA™MiniPrep Plus kit (Zymo Research) and, following the manufacturer's recommendations, diluted in 100 µl of nucleasi free water.
RNA extracted from all samples was quantified by the Qubit 2.0 fluorometer (Thermofisher Scientific), using the Qubit ™ RNA High Sensitivity (HS) kit, according to manufacturer's instructions.Concerning purity, all RNA samples showed absorbance ratios ($A_{260nm}/A_{280nm}$ and $A_{260nm}/A_{230nm}$) above 1.9 (data not shown), indicating a high level of purity.

### 2.5.2 cDNA synthesis

Reverse transcription (RT) was performed using 2,5µg of the total RNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermofisher Scientific) with random hexamers and according to the manufacturer's instructions.
A control cDNA sample was made for each organ by pooling the same volume of each cDNA sample.

### 2.5.3 Primers and PCR efficiency (*Eff*)

The genes to be tested as biomarkers were chosen on the basis of the scientific bibliography to date (Brandts et al., 2018; Lacroix et al., 2014; Pont et al., 2016), and are listed in Table 1. In addition, as mRNA expression analysis requires normalization, the first part of this study consisted of identifying the reference genes that is constitutional genes (housekeeping) that do not show variations in gene expression under the experimental conditions.
Two genes, one for the gills and another for the digestive gland, were selected as housekeeping candidates already described in the literature by Brandts et al., 2018:

- β-actin, gene more stable in the gills;
- tubulin, gene more stable in the digestive gland.

Primers were synthesized by Eurofins Genomics (Germany GmbH).
PCR efficiency (*Eff*) was calculating for each primer pair in both organs by making standard curves from serial dilutions of reference cDNA (from 1/50 to 1/800) and using the following formula (Pfaffl, 2001):

$$Eff = 10^{-1/slope}$$

Efficiency of the amplification was determined for each primer pair using serial 5-fold dilutions of pooled cDNA and calculated as E = 10(−1 / s), where s is the slope generated from the serial dilutions (Pfaffl, 2001).
Primer pair specificities were checked both in silico and empirically by BLAST analysis and using melting profiles. BLAST analyses indicated all primers were specific, which was confirmed by melting profiles (data not shown).

### 2.5.4 qPCR Sybr Green

qPCR Sybr Green were performed using the Applied Biosystems 7500 RealTime System. Assays were performed in triplicate using PowerUp™ SYBR™ Green Master Mix (Thermofisher Scientific) according to the manufacturer's instructions.
The PCR program consisted of 2 min of Dual-Lock DNA polymerase activation at 95 °C followed by 40 cycles of 15 s of denaturation at 95 °C, 15 s of annealing at 55-60 °C and 1 min of elongation at 72 °C. For each sample a melting curve program was performed having the following conditions: 1 cycle 95 °C for 15 sec, 60 °C for 1 min and 95 °C for 15 sec. To minimize technical variation, all samples were analysed on the same run for one gene. Each PCR run included the control cDNA sample and water controls.
Relative gene expression was calculated with the ΔΔCt method (Livak and Schmittgen, 2001).

| Gene name | Abbreviation | Pathway/function | Forward (5' -3') | Reverse (5' -3') | Accession number | Reference |
|---|---|---|---|---|---|---|
| β-Actin | *act* | Housekeeping gene | CGACTCTGGAGATGGTGTCA | GCGGTGGTTGTGAATGAGTA | AF157491.1 | Brandt et al., 2018 |
| α-Tubulin | *tub* | Housekeeping gene | CTTCGGTGGTGGTACTGGAT | AGTGCTCAAGGGTGGTATGG | HM537081.1 | Brandt et al., 2018 |
| Cytochrome P450-1-like-1 | *cyp11* | Phase I biotransformation | TGGTTGCGATTTGTTATGCCCTGGA | GGCGGAAAGCAATCCATCCGTGA | JX885878 | Zanette et al., 2013 |
| Cytochrome P450-3-like-2 | *cyp32* | Phase I biotransformation | CAGACGCGCCAAAAGTGATA | GTCCCAAGCCAAAAGGAAGG | AB479539 | Lacroix et al., 2014 |
| π-glutathione-S-transferase | *π-gst* | Phase II biotransformation | CCTGAAACCAACCAAGGGTTACAT | TGGACTCCTGGTCTAGCCAACACT | AF227977/AF527010 | Lacroix et al., 2014 |
| P-53 tumor supressor-like | *p53* | Cellular stress response | CAACAACTTGCCCAATCCGA | GGCGGCTGGTATATGGATCT | AY579472/DQ158079 | Lacroix et al., 2014 |
| Heat shock protein 70 | *hsp70* | Cellular tissue repair | CCCTTTCTTCAAGCACACAAGCA | AACTGGTTCCATGGTTCCTCTGAA | AF172607 | Brandt et al., 2018 |
| Cathepsin | *cat* | Immune system | CGCAGCTAATGTTGGCGCC | CTACGGCGATTGGTCCCTG | AF172607 | Brandt et al., 2018 |
| Lysosyme | *lys* | Immune system | TCGACTGTGGACAACCAAAA | GTGACCAATGTACCTCGCCA | AF334662 / AF334665 | Paul-Pont et al., 2016 |

Table 1. Gene name, abbreviation, pathway/function, primer pair and Genbank accession numbers for reference and target genes analyzed in digestive gland and gills of mussel *Mytilus galloprovincialis*

## 2.6 Statistical analysis

The statistical analyses of data were performed using R Core Team software (2021) and Excel 2016 (Microsoft, Silicon Valley, California, USA).

Normality of data set was tested with Shapiro-Wilk test. Then, non-parametric tests were used if the data were not normally distributed.

Beta distribution with 95% confidence intervals was used to verify the variability between the three experimental replicates with respect to the percentages of organisms contaminated by microplastics.

A Kruskal-Wallis test was applied to verify any differences between the experimental groups with respect to the variables "size" and “n. particles MPs/g”. When the Kruskal-Wallis test was significant, Dunn's post-hoc tests were carried out for comparisons between all possible pairs and Bonferroni's correction was applied to it.

Linear regression analysis was used to verify the possible existence of a linear relationship between the number of MPs particles per gram and time.

The Spearman rank correlation test was performed to test any correlation between size of the mussels and the number of MPs.

The analysis with $p < 0.05$ were considered statistically different.

# 3 Results

## 3.1 MPs' qualitative and quantitative analysis

The results of the analyzes carried out for each replicate analyzed are detailed below.

| EXPERIMENTAL GROUP (replica 1) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **N. contaminated organisms** | **N. contaminated organisms (%)** | **N. MPs found** | **Average n. total MP particles/organisms** | **Average n. total MP particles/g** | **Granules (%)** | **Fibers (%)** | **Fragments (%)** |
| **Group T0** | 20/20 | 100 | 182 | 2,40 | 9,1 | 36 | 60 | 4 |
| **Group T2** | 19/20 | 95 | 63 | 0,79 | 3,1 | 11 | 87 | 2 |
| **Group T7** | 15/20 | 75 | 28 | 0,37 | 1,4 | 7 | 89 | 4 |

**Table 2.** "Laboratory uptake and clearance experiment" summary data of the experiment - replica 1. Group T0: 20 organisms collected at the end of the 3 days of exposure to MPs; Group T2: 20 organisms collected at the end of the 2 days of purification (microbiological purification); Group T7: 20 organisms collected at the end of the 7 days of purification (experimental purification for MPs).

| EXPERIMENTAL GROUP (replica 2) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | N. contaminated organisms | N. contaminated organisms (%) | N. MPs found | Average n. total MP particles/organisms | Average n. total MP particles/g | Granules (%) | Fibers (%) | Fragments (%) |
| **Group T0** | 20/20 | 100 | 217 | 10,85 | 2,15 | 37 | 61 | 2 |
| **Group T2** | 14/20 | 70 | 38 | 1,9 | 0,45 | 16 | 71 | 13 |
| **Group T7** | 13/20 | 65 | 20 | 1 | 0,17 | 0 | 95 | 5 |

**Table 3.** "Laboratory uptake and clearance experiment" summary data of the experiment - replica 2. Group T0: 20 organisms collected at the end of the 3 days of exposure to MPs; Group T2: 20 organisms collected at the end of the 2 days of purification (microbiological purification); Group T7: 20 organisms collected at the end of the 7 days of purification (experimental purification for MPs).

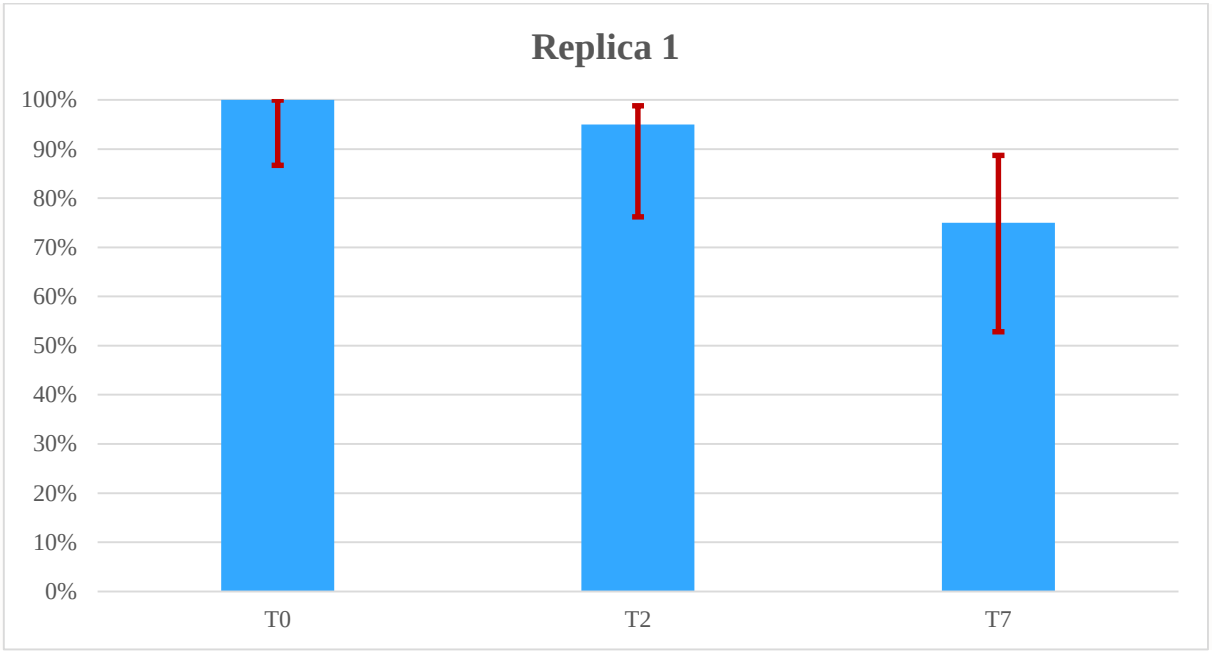
| EXPERIMENTAL GROUP (replica 3) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | N. contaminated organisms | N. contaminated organisms (%) | N. MPs found | Average n. total MP particles/organisms | Average n. total MP particles/g | Granules (%) | Fibers (%) | Fragments (%) |
| **Group T0** | 20/20 | 100 | 203 | 10,15 | 1,97 | 17 | 83 | 0 |
| **Group T2** | 15/20 | 75 | 26 | 1,3 | 0,24 | 8 | 88 | 4 |
| **Group T7** | 11/20 | 55 | 27 | 1,25 | 0,26 | 0 | 89 | 11 |

**Table 4.** "Laboratory uptake and clearance experiment" summary data of the experiment - replica 3. Group T0: 20 organisms collected at the end of the 3 days of exposure to MPs; Group T2: 20 organisms collected at the end of the 2 days of purification (microbiological purification); Group T7: 20 organisms collected at the end of the 7 days of purification (experimental purification for MPs).

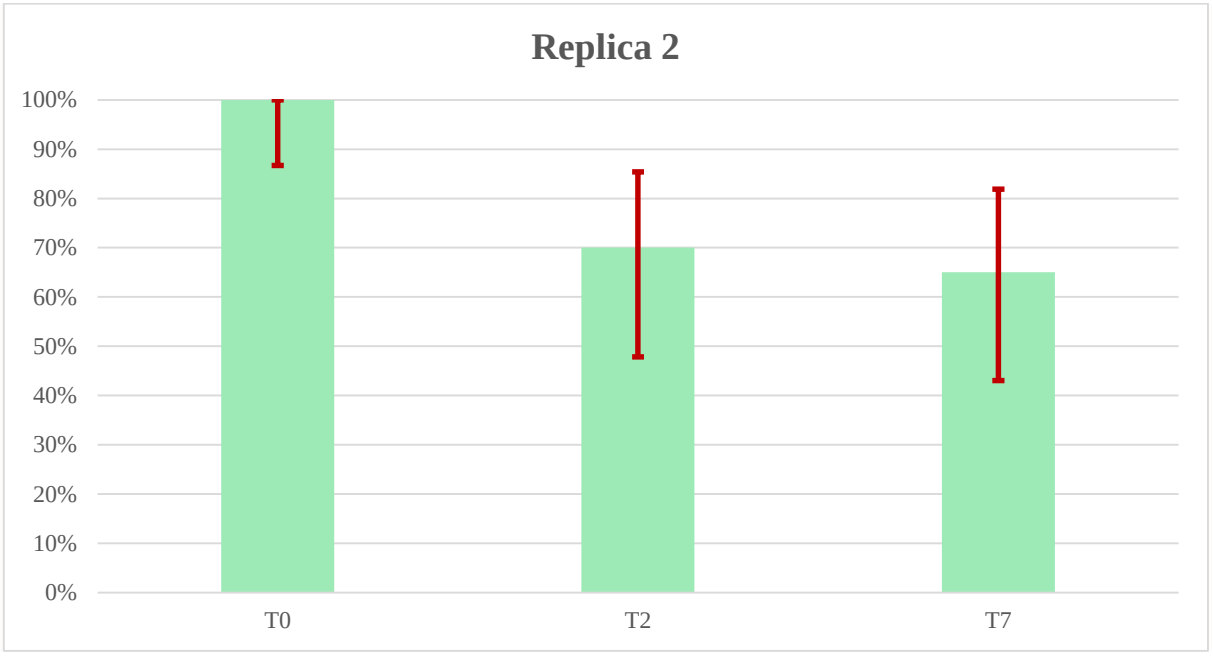
By analyzing the percentages of organisms contaminated by microplastics (Table 5; Graphs 1, 2 and 3), with the relative 95% confidence interval, calculated by means of the Beta distribution, in each of the 3 replicates it is possible to observe a variability between replicates that is not statistically significant, as the 95% confidence intervals are comparable. This means that the presence of the experimental error in our experiment does not interfere with the results obtained, as the precision of an experiment is nothing more than the controlled variability of the results between one replicate and another.

| | N. contaminated organisms | N. contaminated organisms (%) | l.c.i. 95% | l.c.s. 95% | N. MPs found | Average n. total MP particles/g |
|---|---|---|---|---|---|---|
| **EXPERIMENTAL GROUP (replica 1)** | | | | | | |
| **Group T0** | 20/20 | 100% | 87% | 100% | 182 | 2,40 |
| **Group T2** | 19/20 | 95% | 76% | 99% | 63 | 0,79 |
| **Group T7** | 15/20 | 75% | 53% | 89% | 28 | 0,39 |
| **EXPERIMENTAL GROUP (replica 2)** | | | | | | |
| **Group T0** | 20/20 | 100% | 87% | 100% | 217 | 2,15 |
| **Group T2** | 14/20 | 70% | 48% | 85% | 38 | 0,45 |
| **Group T7** | 13/20 | 65% | 43% | 82% | 20 | 0,17 |
| **EXPERIMENTAL GROUP (replica 3)** | | | | | | |
| **Group T0** | 20/20 | 100% | 87% | 100% | 203 | 1,97 |
| **Group T2** | 14/20 | 95% | 48% | 85% | 26 | 0,24 |
| **Group T7** | 11/20 | 55% | 34% | 74% | 27 | 0,26 |

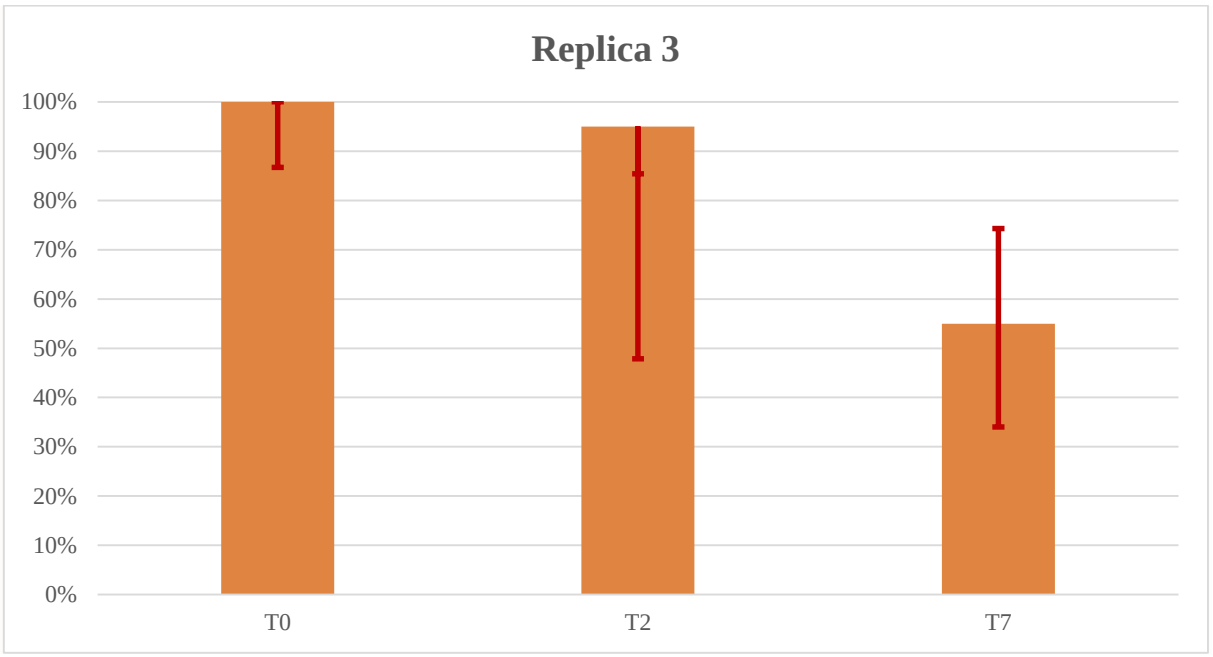
**Table 5.** Summary table of the progress of organisms contaminated by microplastics in the three experimental replicates with l.c.i. and l.c.s.




**Graph 1.** Graphical representation of the percentage of organisms contaminated by microplastics in replica 1 with 95% confidence interval.




**Graph 2.** Graphical representation of the percentage of organisms contaminated by microplastics in replica 2 with 95% confidence interval.




**Graph 3.** Graphical representation of the percentage of organisms contaminated by microplastics in replica 3 with 95% confidence interval.

On the values relating to “n. MPs/g particles” a linear regression analysis was performed to verify the possible existence of a linear relationship between the number of MPs particles per gram and time. From table 6 and the regression plot (Graph 4) it is possible to highlight that there is a statistically significant decrease (p value 2.5E-14) in the presence of the number of microplastic particles found per gram of soft tissue of the analyzed mussels, in fact we have as average values in the 3 experimental groups:

- Group T0: 2.17 MPs/g;
- Group T2: 0.49 MPs/g;
- Group T7: 0.27 MPs/g.

This decrease can be seen already after 2 days of purification and is even more evident after 7 days of purification.

***Regression statistics***

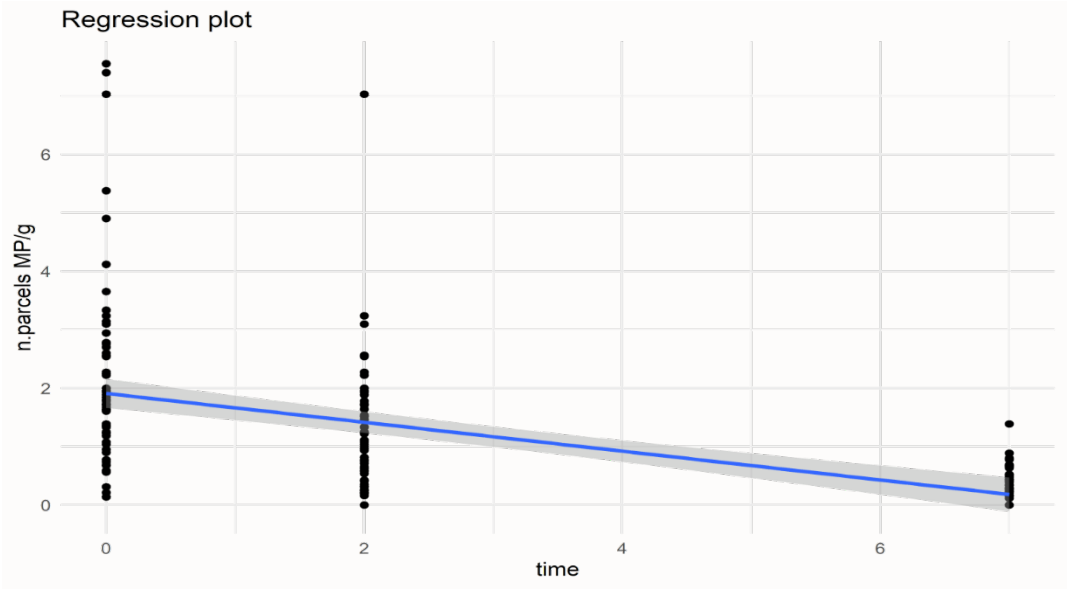
| | |
|---|---|
| Multiple R | 0,528397032 |
| R squared | 0,279203423 |
| R squared corrected | 0,275154004 |
| Standard error | 1,174482941 |
| Remarks | 180 |

**VARIANCE ANALYSIS**

| | *gdl* | *SQ* | *MQ* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 95,10896414 | 95,10896414 | 68,94900845 | 2,4687E-14 |
| Residue | 178 | 245,535012 | 1,37941018 | | |
| Total | 179 | 340,6439761 | | | |

| | *Coefficients* | *Standard error* | *Stat t* | *Significance value* | *Inferior 95%* | *Superior 95%* |
|---|---|---|---|---|---|---|
| Intercept | 1,909368033 | 0,124986101 | 15,27664285 | 3,29246E-34 | 1,662722849 | 2,156013216 |
| TIME | -0,246915542 | 0,029736128 | -8,303553965 | 2,4687E-14 | -0,305596248 | -0,188234835 |

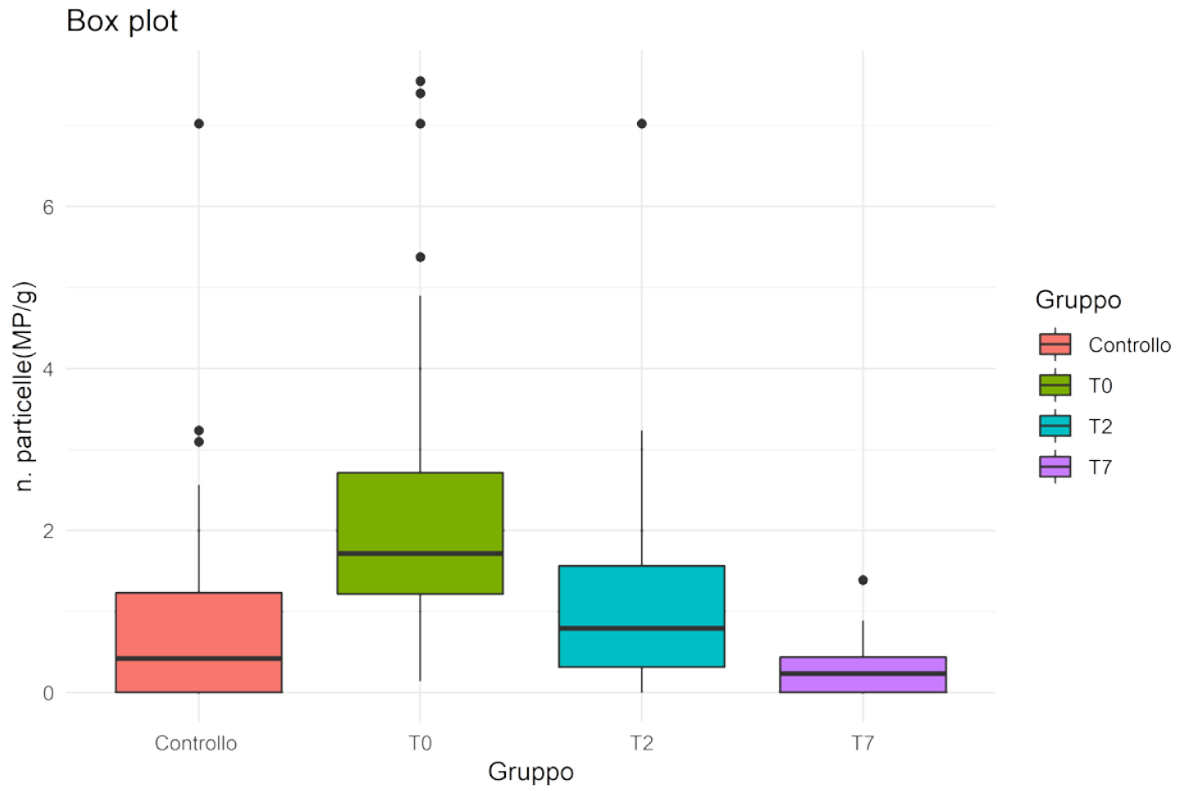
**Table 6.** Summary table of linear regression analysis for verifying a linear relationship between the time variables (T0, T2 and T7) and n. particlesMPs/g.




**Graph 4.** Graphical representation of trend of n. particles MPs/g in three experimental replicates.

To verify the differences in the experimental groups (control, T0, T2 and T7 group) with respect to the variable "n. particles MPs/g" (Graph 5) a Kruskall-Wallis test was performed. Given the significance of this comparison (p.value = 0.0000005) it was possible to apply the related Dunn's post-hoc tests (with Bonferroni correction). Among all the possible couples in comparison, a statistically significant difference was highlighted between:

- ***Control group*** and ***T0 group*** (p. value: 1,23336E-06): this data indicates that the T0 group is correctly contaminated experimentally with microplastics compared to the control group;
- ***T0 group*** and ***T7 group*** (p. value: 0.007125783): data that indicates a good capacity of the mussels after 7 days to be able to properly purify the microplastics accumulated within their soft tissue.




**Graph 5.** Graphical representation of the differences in the control groups, T0, T2 and T7 of the n. particles MPs/g.

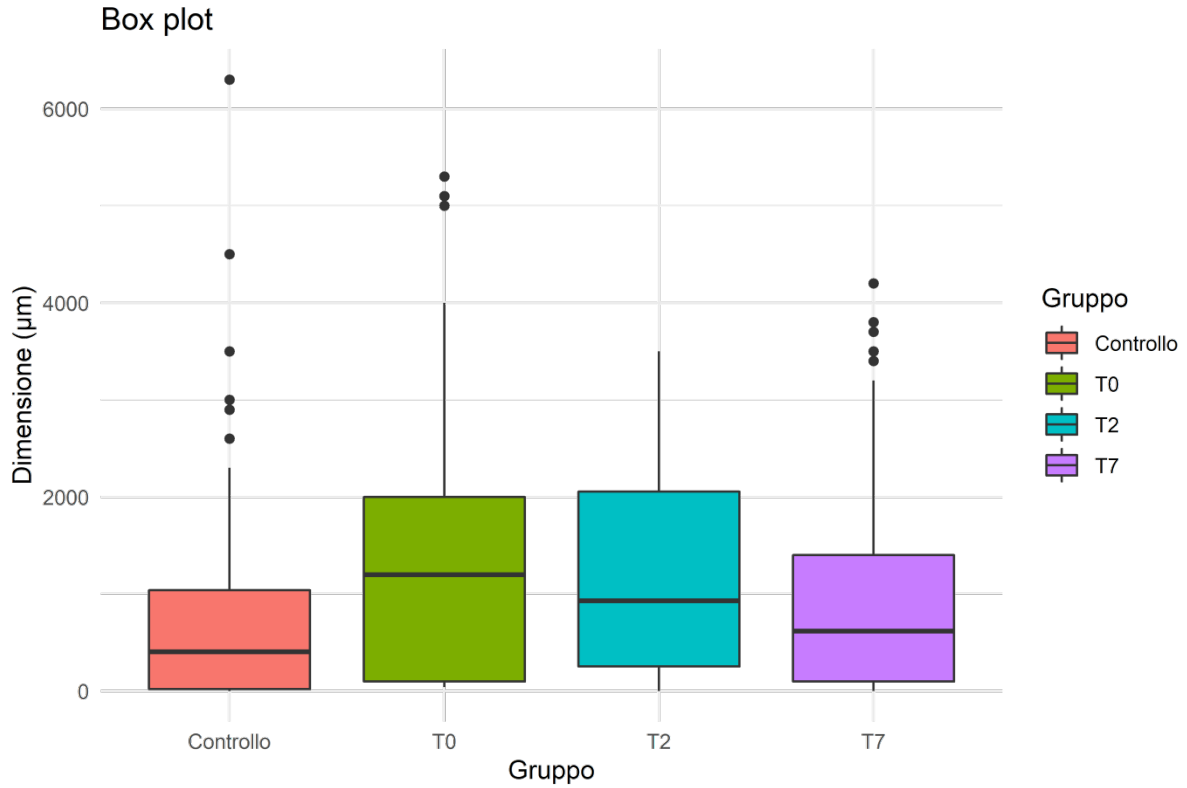
From the statistical comparison of the experimental groups (control, T0, T2 and T7 group) with respect to the variable "size" of the microplastic particles (Table 7 and Graph 6) carried out through the Kruskall-Wallis test, a statistically significant difference is highlighted (p.value = 1.08E-18).

From the application of Dunn's post-hoc tests, with Bonferroni correction, result statistically significant comparisons between:

- ***Control group*** vs ***T0 group*** (p. value: 4.42E-07) and ***Control group*** vs ***T2 group*** (p. value: 0.0001). From this data we can deduce that the frequency in the control group of microplastic particles of size 50-100 µm (most represented size class) is statistically different from that found in groups T0 (most present size class: 1000-2000 µm) and T2 (class most present dimensional: <1000 µm);
- ***T0 group*** and ***T7 group*** (p. value: 0.007048). This seems to indicate that the frequency in the T0 group of microplastic particles of size 1000-2000 µm (most represented size class) is statistically different from that present in the T7 group (most present size class: <1000 µm).

| | **10 - 50 µm** | **50 – 100 µm** | **200 µm** | **100 – 500 µm** | **< 1000 µm** | **1000 - 2000 µm** | **2000 - 3000 µm** | **> 3000 µm** |
|---|---|---|---|---|---|---|---|---|
| **Control group** | 1% | 70% | 0% | 1% | 15% | 7% | 3% | 3% |
| **T0 group** | 0% | 0% | 6% | 1% | 18% | 43% | 24% | 7% |
| **T2 group** | 2% | 13% | 2% | 0% | 33% | 17% | 31% | 2% |
| **T7 group** | 1% | 5% | 0% | 3% | 51% | 20% | 11% | 9% |

**Table 7.** Summary table of the percentage presence of the various size classes of microplastic particles in the comparison groups (Control, T0, T2 and T7 group).

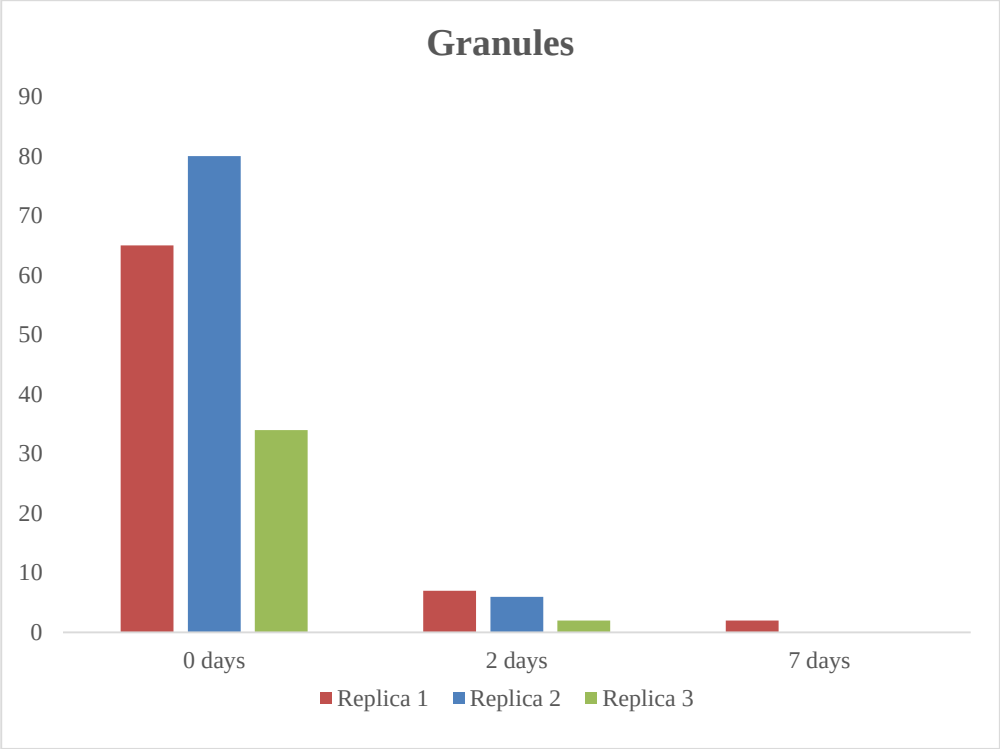



**Graph 6.** Graphical representation of the differences in the control groups, T0, T2 and T7 of the size of the microplastic particles.

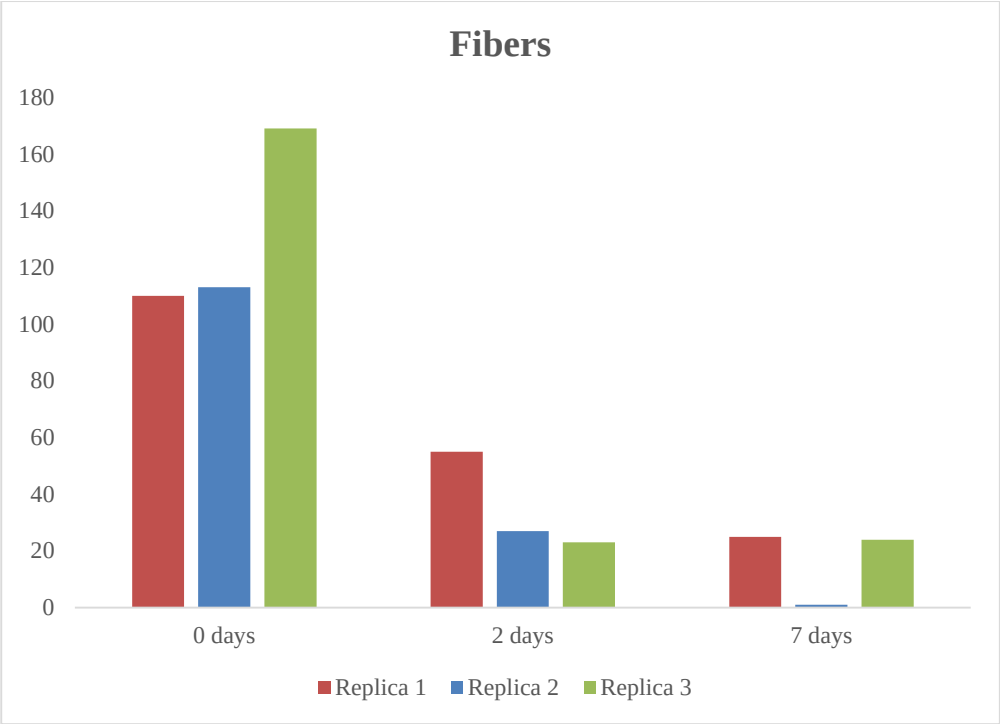
The Chi-squared (corrected) test was performed on the variable “type of microplastics” (Table 2, 3, 4) in order to verify the presence of any differences between the experimental groups (T0, T2 and T7) and the types of microplastic particles in each of the 3 experimental replicates statistical analysis revealed significant differences in all replicates (Replica 1 Chi-squared (corrected) test: 22,35968/p.value: 0,000784; Replica 2 Chi-squared (corrected) test: 26,54112/p.value: 0,000975; Replica 3 Chi-squared (corrected) test: 25,53073/p.value: <0,0001).

Furthermore, from Graphs 7, 8 and 9 it is possible to observe the different decrease that the number of each type of microplastic under examination has undergone in the three experimental

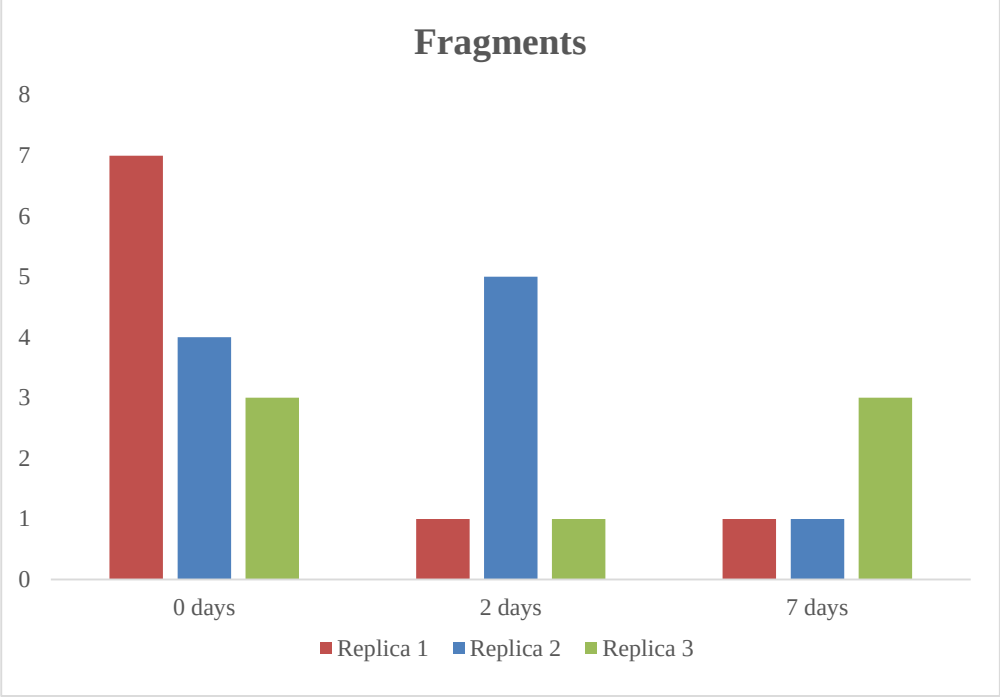
replicas. The type of microplastics that seems to be eliminated more effectively by mussels, both after 2 and 7 days of purification, are granules, followed by filaments and fragments.




**Graph 7.** Graphical representation of the number of granules in the three experimental replicates.




**Graph 8.** Graphical representation of the number of fibers in the three experimental replicates.



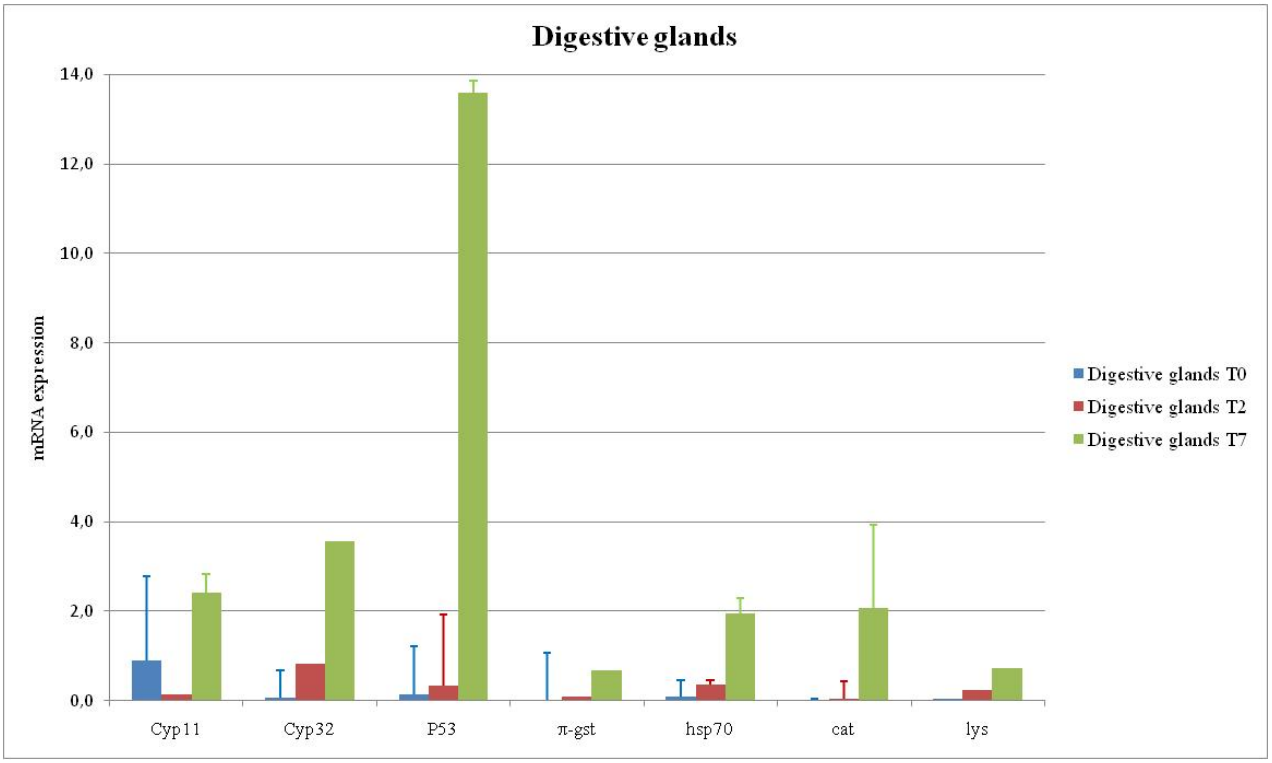

**Graph 9.** Graphical representation of the number of fragments in the three experimental replicates.

Finally, the Spearman's rank correlation rho test was performed to evaluate whether from a statistical point of view there was a positive correlation between the size of the mussels (expressed in soft tissue weight) and the number of microplastic particles present in their tissues. There was no significant correlation between the two variables (p.value = 0.6313).

## 3.2 Gene expression analysis

As shown in Graphic 10 and 11, 7 markers known to be involved in pollutant response were tested in mussel gills and digestive glands.

Concerning expression of target genes in digestive glands of *M. galloprovincialis* (Graph.10), transcriptional levels of genes associated with biotransformation and detoxification processes (cyp11, cyp32, π-gst) were basically increased in relation to control group in mussels' group after 7-days purification time (T7 groups). Instead, the expression of π-gst gene were unaltered in digestive gland of all mussels' groups. Also the expression's level of p53 gene associated to DNA damage repair and hsp70 gene related to cell-tissue repair were increased in digestive gland of mussels' after 7-days purification time (T7 groups) in comparison to the control group. At last, regarding immune genes cat and lys, mRNA levels of lys were unaltered in all analyzed mussels' groups, while the cat gene was increased in mRNA abundance in digestive gland of mussels after 7-days purification time (T7 groups) in relation to control group.

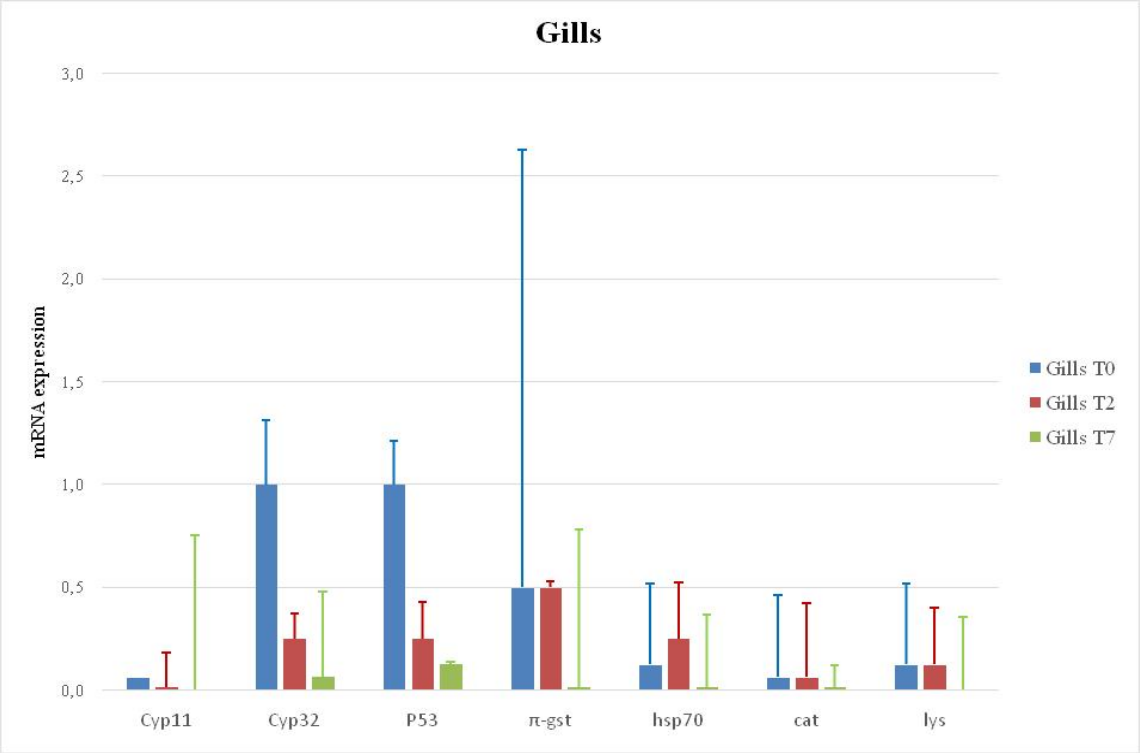



**Graph 10**. mRNA relative levels in digestive glands for target genes as a function of sampling times. Error bars = standard error of the mean.

In gills, mRNA levels of cyp11 gene were unaltered in all mussels' groups. Instead an increase in mRNA abundance of cyp32 was found in mussels after 3-days MPs exposure compared to control group (Graph. 11).

Transcriptional levels of π-gst and p53 in gills increased in the 3-days MPs exposure group (T0 group) in comparison to control group. Furthermore, mRNA levels of π-gst increased also in the 2-days purification time group (T2 group).

The expression levels of hsp70 in gills appeared unaltered, when compared to control group. Finally also mRNA abundance of lys and cat remained unaltered in comparison to control group.




**Graph 11**. mRNA relative levels in gills for target genes as a function of sampling times. Error bars = standard error of the mean.

# 4 Discussion

Exposure experiments are regarded as effective methods to study the uptake, accumulation and toxicity of contaminants.

The ingestion and biological effects of microplastics have been tested in many previous studies (Birnstiel et al., 2019; Rist et al., 2019; Hu et al., 2016; Avio et al., 2015; Van Cauwenberghe et al., 2015; De Witte et al., 2014).

Considering the limitations imposed by the common approach of using a single type of microplastic, in the present study we used microplastics of three different shapes to simulate the microplastic type observed in the real environment, such as in the study of Qu et al., 2018.

In the exposure experiments, the accumulation of microplastics was observed in all mussels.

The replicates, conducted under the same experimental conditions, did not show statistically significant variability, therefore the experimental error did not interfere with the results obtained.

From the linear regression analysis, used to verify the possible existence of a relationship between the variables "number of MPs particles per gram" and "time", it was possible to detect a considerable variability in terms of the presence of microplastics found in the soft tissue of mussels analyzed among the samples analyzed at time 0.

This variability leads to a rather low corrected R-squared value (equal to 0.275) and could be explained by probably too short contamination times, which therefore did not allow all mussels to be able to contaminate themselves with microplastics. In fact, even other studies (Rist et al., 2019), where very short exposure times (10, 20 and 40 minutes) were performed, a greater presence of microplastics used for contamination of organisms (microspheres) was observed in the water of the exposure phase, probably because three exposure times were too short to allow mussels to accumulate these contaminants.

After 7 days of purification (experimental purification) it was possible to highlight a statistically significant decrease (p value 2.5E-14) in the presence of the number of microplastic particles found per gram of soft tissue of the analyzed mussels (Group T0: 2.17 MPs/g; Group T2: 0.49 MPs/g; Group T7: 0.27 MPs/g).

Instead, no statistically significant differences emerged between the T0 and T2 groups (p.value: 1), indicating that the two-days depuration time may not have been long enough to completely eliminate the microplastic particles or that MP could have been translocated to other tissues, or even to the circulatory system (Birnstiel et al., 2019; von Moos et al., 2012; Browne et al., 2008). Also, in other studies (Birnstiel et al., 2019) the short purification times were not sufficient to completely eliminate microplastics in bivalve mollusks.

Longer purification times (7 days) have been tested in other studies and in other bivalves (Xu et al., 2016; Ribeiro et al., 2017) suggesting that even a longer depuration period will not be enough to completely recover bivalves from MP exposition. Therefore, it is noteworthy that depuration can minimize the effects caused by MP contamination, even if it does not reach a 100% reduction (Birnstiel et al., 2019).

Statistically significant differences were found between the T0 group and the T7 group (p. value: 0.007048) as regards the size class of microplastics found within the analyzed mussels. In the T0 group the most represented size class was 1000-2000 µm unlike in T7 group microplastics <1000 µm were more present. These results could explain the ability of bivalve mollusks to eliminate larger microplastic particles more easily and in a shorter time. In fact, even in other bivalves, such as *Mytilus edulis* and *Crassostrea gigas*, a greater purification capacity of larger microplastic particles has been found (Browne et al., 2008; Van Cauwenberghe and Janssen, 2014).

Furthermore, smaller sizes of microplastics also show greater toxicity to organisms like Copepoda adults and offspring (Lee et al., 2013). Therefore, great attention should be paid to the size effects of microplastics ingested by mussels, especially for those microplastics in smaller size classes (Qu et al., 2018).

Microplastics that seems to be eliminated more effectively by mussels, both after 2 and 7 days of purification, are granules, followed by filaments and fragments. Probably the spherical size of the granules allows them not to be strongly retained within the gills of the hepatopancreas of the mussels, which therefore are able to get rid of them more easily; in fact, we observe an almost total absence of this type of microplastics in the analyzed organisms in all three experimental replicas

after 7 days of purification (Birnstiel et al., 2019; De Witte et al., 2014). This result appears to be in contrast with the study by Qu et al., 2018 in which it was instead highlighted that beads were more easily ingested by mussels in laboratory conditions, because were smaller than fibers, they could be transferred and accumulated inthe digestive system and the haemolymph (Browne et al., 2008; von Moos et al., 2012).

Filaments instead, due to their geometry, remain better trapped in the gills and hepatopancreas of mussels and cannot be easily removed through the filtration animals (Renzi et al., 2018; De Witte et al., 2014). On the other hand, fragments didn't decrease in the three experimental replicas.

Furthermore, no significant correlation was highlighted between the two variables "mussel size" (expressed in soft tissue weight) and the "number of microplastic particles present in tissues", confirming that the ability to accumulate microplastic particles inside bivalve, as well as the ability to eliminate them, is not strictly correlated with animal size, as also reported in other experiments (Birnstiel et al., 2019).

As reported by Qu et al., 2018, the present microplastics exposure experiments are different from the real conditions of microplastics in the environment even though some relationship between microplastic in mussels and in exposure water could be observed.

These differences mainly depend on two factors:

a) microplastics present in the environment could have different physico-chemical properties such as shapes, sizes, colors, compositions, and additives (Lambert et al., 2017), therefore it is difficult to emulate in the laboratory a real contaminating mixture.

b) Microplastics accumulation in marine environment occurs over a long period while the exposure experiments are shorter, therefore great efforts are needed to simulate this aspect in exposure studies (Qu et al., 2018).

Concerning gene expression analysis, in this study, MPs induce changes in expression of genes associated with biotransformation (cyp11, cyp32 and gst) in digestive gland of *M. galloprovincialis*, in contrast to the results of other authors (Oliveira et al., 2013; Lacroix et al., 2014; Pinsino et al., 2017). Specifically, increasing mRNA levels of cyp11 and cyp32 genes were observed in the digestive

glands of mussels subjected to 7 days of purification after being contaminated for 3 days with microplastics. Expression of gst suffered no changes, in agreement with the findings of Oliveira et al. (2013), that reported no modulation of this gene activity after exposure to polyethylene microplastics in *Pomatoschistus microps*.

In the digestive glands of mussels subjected to 7 days of purification was also observed an increased transcriptional level of hsp70 indicate an induction of de novo synthesis of these proteins (Brandts et al., 2018); and of cat gene, one of the most over-regulated in response to pollutants such as metals and organic compounds (Gomes et al., 2014).

In gills, cyp32 mRNA levels showed an increase in mussels exposed to MPs (T0 group), suggesting an activation/increase of phase I reactions of biotransformation in mussels contaminated compared to control group. Our results, in accordance with other authors (Zanette et al., 2013, Lacroix et al., 2014), confirm the involvement of cyp32 gene in mussel biotransformation processes and its potential as a biomarker.

Also the expression of π-gst gene in gills was observed increased in mussels exposed to MPs (T0 group), in agreement with Lacroix et al. 2014.These results suggest an increase in biotransformation phase II processes, which highlight the potential of π-gst mRNA level as a pollution biomarker (Lacroix et al.,2014). Other authors, such as Fernández et al. 2012, have also examined the activities of GST enzymes in *Mytilus* spp. in response to pollutants, reporting instead a decrease in phase II biotransformation processes in organisms exposed to pollutants.

In addition, in gills of contaminated mussels (T0 group) has been observed an increase p53 mRNA level, considered as a cellular stress marker in mussels (Estévez-Calvar et al., 2013). This up-regulation of expression observed for p53 gene could correspond to a better modulate of the transcription of genes involved in DNA repair suchas Growth Arrest- and DNA Damage inducible gene 45 alpha (*gadd45a*) (Ruiz et al., 2012).

In gills, transcriptional levels of hsp70 have remained unchanged both after exposure to MPs and after purification processes. As reported in the scientific bibliography, the upregulation of hsp70 expression can be considered as a general rule in aquatic organisms exposed to pollutants (Piano

et al., 2004; Ivanina et al., 2009) although some authors have highlighted a its downregulation in *M. galloprovincialis* following exposure to several contaminants (Izagirree al., 2014; Brandts et al., 2018).

The representative genes of the immune system, cat and lys, remained unaltered in the group of contaminated mussels when compared to control group. Unlike what is reported by Brandts et al. 2018, whose overall results indicate a weakening of the studied organisms' immune defense in organisms subjected to higher doses of contaminant (polystyrene nanoplastics) in combination with carbamazepine. The authors based on their findings speculate that this weakened condition could diminish mussels' bactericidal capacity, leading to a higher sensibility to foreign aggressions.

The present study demonstrated that *M. galloprovincialis* are sensitive to microplastics as they are pollutants. Gene expression data showed that the gills is the organ in which they have been observed the most differences in contaminated mussels' group with respect to the control group. This organ indeed is a key element for the uptake of waterborne toxicants during filtration processes and to temporarily accumulate high concentrations of contaminants before their probable transfer to the digestive gland and other tissues (Bustamante et al., 2012). Furthermore, gills are the organ of first contact with the waterborne pollutants (Franco-Martinez et al., 2016) and one of the main detoxification organs in this species (Gomes et al., 2014), which could justify the more pronounced gene expression alteration in this organ. In particular, the microplastics altered the expression of genes associated with gills' biotransformation, DNA damage and cell-tissue repair. Therefore, the results of the present work confirm; firstly, that cyp32, π-gst and P53 are transcript biomarker candidatesin mussel gills, in accordance with Lacroix et al. 2014; and secondly, that gills are an organ of choice for biomonitoring studies (Manduzio et al., 2004).

In conclusion, the result of present study demonstrates that (a) the purification processes can significantly reduce MPs contamination in *Mytilus galloprovincialis*, though longer depuration periods need to be tested, in order to assess the possibility of an even greater reduction in MPs contamination; (b) the use of qPCR technology and mRNA levels as early-warning biomarkers in marine monitoring programs are very useful and innovative.

# 5 Attachments

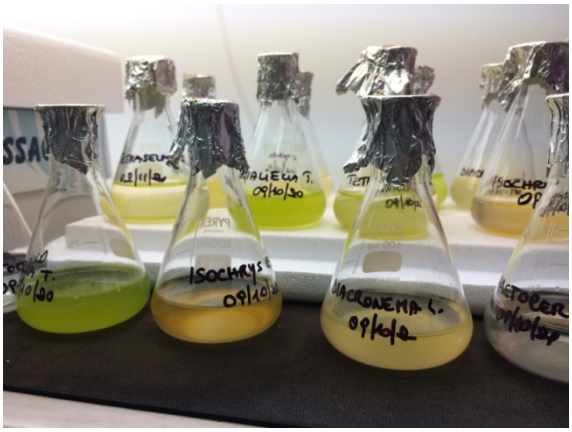
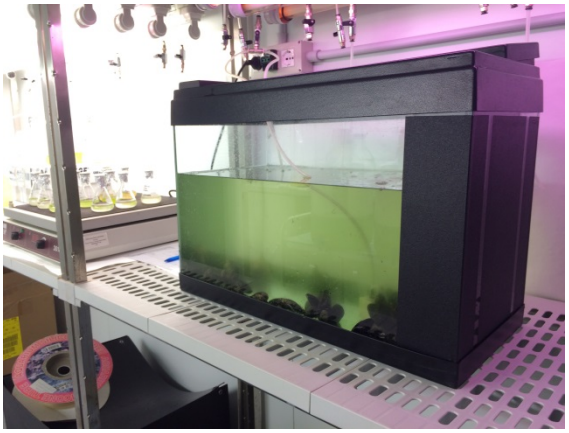


**Image 1.** Algal cultures used to feed mussels.



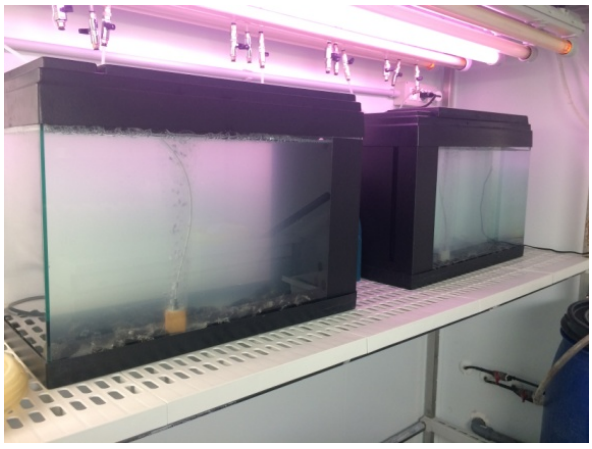


**Image 2.** Mussels in the glass tanks during the experiment.




**Image 3.** MPs' qualitative and quantitative analysis.

**Image 4.** qPCRSybr Green analysis

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