Microlitter pollution in the marine environment and preliminary evidences of in vitro cytotoxic effects on two Mediterranean commercial fish species

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Abstract

Marine litter, which is composed mainly of plastics, is recognized as one of the most serious threats to marine ecosystems and a global environmental concern. Microplastics (MPs) densities were estimated in all environmental compartments: marine organisms are highly exposed to and ingest them, resulting in disruption of biological functions. Ecotoxicological approaches have also started elucidating the potential severity of MPs in controlled laboratory studies, but the commercially-available and pristine materials employed hardly reflect the actual composition of the environmental litter, which can be contaminated by chemical pollutants or biological agents. Building on the lack of research employing marine environmental MPs or microlitter as a whole, we characterized the quantity and quality of litter in the coastal epipelagic and in the digestive tract of two commercially-relevant fish species, and exposed primary cell cultures of mucosal and lymphoid organs to marine microlitter. A concentration of 0.30 ± 0.02 microlitter items m⁻³ was found in the water column of the Northern Tyrrhenian sea. μFT-IR analysis revealed that particles of plastic origin, namely polypropylene, HDPE and polyamide, were present in 100% and 83.3% of M. merluccius and M. barbatus stomachs, respectively, which overall ingested 14.67 ± 4.10 and 5.50 ± 1.97 items. Microlitter was confirmed as a vector of bacteria, fungi and flagellates. Lastly, and for the first time, the apical end-point of viability was significantly reduced in splenic cells exposed in vitro to two microlitter conditions. Considering the role of the spleen in the mounting of adaptive immune responses, our results warrant more in-depth investigations for clarifying the actual susceptibility of the biota to anthropogenic microlitter.

Keywords

Marine microlitter; Bioindicators; Cytotoxicity; In vitro approaches; Primary cell cultures; Biological agents
Highlights

- 0.30 ± 0.02 microlitter items m$^{-3}$ was found in the coastal epipelagic Northern Tyrrhenian sea
- 14.67 ± 4.10 and 5.50 ± 1.97 items were retrieved from hake and mullet stomach contents
- A subsample of the ingested microlitter was of plastic origin
- Microlitter was validated as a carrier of bacteria, fungi and flagellates
- Splenic cells exposed to both microlitter conditions for 72 hours suffered cytotoxicity
**Figure/Table captions**

**Fig. 1** Map of sampling sites - Study area with indications of 250 µm net tows site locations, haul route and bathymetry.

**Fig. 2** A-E Microlitter collected from water column - Some examples of microlitter particles collected from the water column by 250 µm net tows (SUP1-SUP4).

**Fig. 3** Quali-quantitative characterization of anthropogenic litter collected from the water column - A) Particle density of the micro- and macrolitter fractions per net tow. Average litter density is reported as mean ± SE. B) Cumulative microlitter density per type per net tow. C) Size class distribution per microlitter shape type. D-F) Percentage of color abundance per microlitter shape type.

**Fig. 4** Microlitter retrieved from the digestive tract of fish - A) White fragment from *M. barbatus*. B) Black filament from *M. merluccius*. C) Green fragment from *M. merluccius*.

**Fig. 5** Quali-quantitative characterization of microlitter retrieved from fish stomach contents - A) Microlitter particle abundance distribution per species (log y scale). Whiskers plotted according to the Tukey method. B) Cumulative particle abundance per type. C) Size class distribution per microlitter shape type per species. D-G) Percentage of color abundance per microlitter shape type per species.

**Fig. 6** μFT-IR spectra - Spectra of randomly selected representative samples of microlitter retrieved from fish stomach content. Matching with reference substance as per the Open Specy open source database. *r*: Pearson’s correlation coefficient as measure of linear correlation between data sets.

**Fig. 7** A-D Microlitter as a vector of biological agents - Examples of microorganisms observed in cell suspensions following a 72-hour incubation period with microlitter. A) European hake splenic cell, negative control. B-D) Grey, white and black arrowheads indicate bacilliform bacteria, unicellular fungi and flagellates, respectively. Scale bar: 10 µm.

**Fig. 8** Quantification of ATP levels as a proxy of cell viability - ATP data distribution per species, time, organ and treatment. Statistical significance as per one-way ANOVA followed by Tukey’s HSD *post hoc* test. *: *p* < 0.05

**Fig. 9** Comparison of microlitter densities retrieved from representative scientific literature with data herein presented. Color-coded boxes indicate means ± SD items m⁻³.

**Table 1** Sampling details - Summary of the experimental campaign with hauls and net tows details.

Time is expressed as UTC/GMT +2:00.
Table 2 Full statistical details of the rank-based nonparametric Kruskal-Wallis tests performed on microbial counts per species and organ. ns: non significant.

Table S1 Water column microlitter abundance and density (items m$^{-3}$) per color per net tow.

Table S2 Water column microlitter size classes per particle type per net tow.

Table S3 Full statistical details of the one way ANOVA tests performed on cytotoxic data of A) *Merluccius merluccius* and B) *Mullus barbatus*, per time and organ. DFn: degrees of freedom in the numerator; DFd: degrees of freedom in the denominator; F: test statistic for ANOVA; ges: generalized eta squared.
1. Introduction

Coastal areas are subject to an exponential increase in population density and the development of impacting human activities, e.g. industries, tourism, recreational activities, fishing and aquaculture. As a consequence, they can be affected by both sporadic and continuous pollution events, with consequences on all compartments, and are thus considered “hotspots” of contamination (Cole et al., 2011).

Marine litter, defined as “any anthropogenic manufactured, or processed solid material (regardless of size) discarded, disposed of, or abandoned in the environment, including all materials discarded into the sea, on the shore, or brought indirectly to the sea by rivers, sewage, storm water, waves, or winds” (UNEP, 2016), is one of the most serious threats to marine ecosystems and a global environmental concern. It includes glass, metal, cardboard and textiles items (Löhr et al., 2017) as well as anthropogenic particles produced by industrial activities (e.g., coal-fired power plants) and transport emissions (Piazzolla et al., 2020) but Tekman et al. (2021) revealed that plastic accounts for the 66-79% of the global litter composition.

Annual global plastic production accounted for 348 million tonnes in 2018 (Association of Plastic Manufacturers, 2018): about 1.3-3.1% of these (5-12 million tonnes year-1) reach the Oceans (Jambeck et al., 2015), but the total amount of floating plastic was estimated at 0.3 million tons (van Sebille et al., 2015). In addition to primary microplastics (MPs), i.e. particles that are purposefully manufactured of microscopic sizes < 5 mm, the vast majority of marine litter is subject to degradation by abiotic (UV radiation, mechanical abrasion, temperature) and biotic (microbiological depolymerization) agents, resulting into secondary MPs (Ru et al., 2020; Thompson et al., 2004). Their chemico-physical properties, such as type of polymer, density, size, shape, internal geometry and color, influence their transport, buoyancy and sinking as well as rates of ingestion and removal by aquatic organisms (Kowalski et al., 2016; Nguyen et al., 2020; Shim et al., 2018).

Due to their small size, MPs are bioavailable for a variety of taxa (e.g. Cole et al., 2013; Gomiero et al., 2018; Lusher et al., 2013; Pittura et al., 2018) and can either be mistaken with
or selectively chosen instead of food (Clark et al., 2016; Moore, 2008), with demonstrated impacts. Once ingested, MPs can affect biological functions and tissue integrity of marine organisms (Cole et al., 2015; Pedà et al., 2016; Sussarellu et al., 2016). Moreover, MPs can be potential carriers of pollutants (Amelia et al., 2021; Guo and Wang, 2019) and can be colonized by microbial pathogens, transferring them along the trophic web (Caruso, 2019; Casabianca et al., 2019). Due to its geographical and oceanographical features, the Mediterranean Sea is regarded as an accumulation zone for marine litter, with marine litter densities comparable to those of the five subtropical gyres (Cózar et al., 2015; UNEP/MAP, 2015; Van Sebille et al., 2020).

Ecotoxicological and physiological impacts of MPs have not received as much attention and, to the best of our knowledge, no data exist about cytotoxic effects caused by field-collected MPs to cell cultures from fish mucosal and lymphoid organs. The aim of the present study was hence to characterize microlitter abundance in a coastal area of the Northern Tyrrhenian Sea (Italy) in the water column and in the digestive tracts of selected fish species, as well as to evaluate the biological contamination and the potential in vitro cytotoxicity of environmentally-collected microlitter particles on fish primary cell cultures. The European hake *Merluccius merluccius* (Linnaeus, 1758) and the red mullet *Mullus barbatus* (Linnaeus, 1758) were chosen as models based on biological features, commercial relevance, abundance in the sampling area and their suitability as small-scale plastic pollution bioindicators.
2. Material & Methods

   a. Study area

The research was conducted in the Northern Tyrrhenian Sea (Italy), FAO’s General Fisheries Commission for the Mediterranean (GFCM) Geographical Sub-Area 9 (GSA 9). The experimental campaign fell within the physiographical unit (PU) M. Argentario – Cape Linaro, and included the coastal platform that extends from Santa Severa (42.01676 N, 11.95604 E) to the Tarquinia coastal area (42.22243 N, 11.70495 E). More details about bathymetry and sediment type found in the study area can be found in Mancini et al. (2021).

   b. Experimental campaign

The experimental campaign took place on October 23rd 2020 and extended over a 14-hour period (03:00 - 17:00 UTC/GMT +2:00). Four 3-hour long fishing hauls aimed at macrolitter sampling and four 15-minute long horizontal tows with a 250 µm mesh size net aimed at microlitter sampling were performed at 102-115 m depth and sea surface, respectively (Fig. 2). Table 1 reports time of start, coordinates and depth per each fishing haul and tow. Meteo-marine conditions are reported as per the World Meteorological Organization sea state coding.
Qualitative and quantitative characterization of microlitter in the water column

The 250 µm net was equipped with a metered line, a non-filtering cod-end and a flow meter. Upon deployment, utmost care was taken to ensure the net stretched out correctly. Each tow lasted for 15 minutes. Upon retrieval, the content of the net cod-end was transferred to 500 ml containers and stored at 4 °C until transported to the laboratory. Each sample (SUP1- was visually sorted 5 ml at a time using a stereomicroscope (Leica 8APO). Microlitter was classified in terms of shape (i.e. filament, fragment, film), color and size (A: 250 < x < 500 µm; B: 500 < x < 1000 µm; C: 1000 < x < 3000 µm; D: 3000 < x < 5000 µm; E: x > 5000 µm).

Microlitter abundances are presented as items m⁻³ of seawater ± standard error (SE).

d. Fish sampling

Fish specimens were collected in the same fishing area (October 30th 2020, 01:11:00 PM haul start time, start coordinates 41°59.669 N 11°49.214 E, end coordinates 42°03.0410 N 11°44.4621 E) by means of the bottom trawl net typically known as “volantina” geared with a cod-end mesh size of 50 mm diamond and a vertical opening of 4 m (Sala et al., 2013). *M. merluccius* and *M. barbatus* specimens were within the 17.3-20.2 cm and 10.5-12.7 cm total length ranges, respectively: they were employed for the quali-quantitative characterization of microlitter in stomach contents (n=6 per species) and for cytotoxicity assays (n=3 per species).

Immediately following the opening of the net, specimens of similar within-species sizes were sorted and immediately transferred in ice and kept refrigerated until lab processing.
e. Characterization of microlitter quantity and quality in fish stomach contents

In the laboratory, fish stomachs were sampled and preserved in 75% ethanol until processing. Stomach contents were then placed in a Petri dish and visually sorted using a stereomicroscope (Leica 8APO) to classify microlitter particles in terms of shape, size and color, using the same classification as in section 2c. The microlitter found in the stomach contents was maintained in 50 ml falcon tubes with 0.22 µm-filtered water. Microlitter abundances are presented as mean particles per species ± standard error (items ± SE).

f. μFT-IR analysis

To classify the chemical composition of the microlitter retrieved from fish stomach contents, a representative subsample was analyzed by using Fourier-transform infrared microspectroscopy (μFT-IR). The experiments were performed at the DAFNE Laboratory of INFN (Frascati, Italy) in transmission mode, using a Bruker Hyperion 3000 FTIR microscope equipped with a Globar IR source, a broadband beamsplitter (KBr) and a mercury-cadmium telluride (MCT) detector; the beam size was set at 20x20 µm. Spectroscopic analysis yielded absorbance spectra, which were analysed by using the Open Specy open source database (Cowger et al., 2021) with the Pearson’s correlation coefficient as measure of the linear correlation between the data sets. Spectra visualization and overlay were achieved with SpectraGryph v1.2 using the peak normalization method (i.e. each spectrum highest peak within the visible area was set to 1).

g. Assessment of microlitter cytotoxic effects on fish primary cell cultures

Marine microlitter obtained from the same sampling location of fish were stored in 50 ml falcon tubes in 0.22 µm-filtered sea water until employed for cytotoxicity assays. They were dried, randomly selected and counted under a stereo microscope (average item weight 160 µg).

Isolation and cultivation of fish primary cells were performed according to published standard procedure. Gills (G), head kidney (HK) and spleen (SPL) from *M. merluccius* and *M. barbatus* specimens (n=3) were removed and immersed in cold Hanks Balanced Salt Solution without calcium and magnesium (HBSS), previously adjusted for appropriate sea water osmolarity.
(355 mOsm Kg\(^{-1}\)) with 3M NaCl. Cells were obtained by pushing organs with a plastic pestle in cold HBSS through 100 and 40 µm nylon mesh strainers and washing by centrifugation (10 min, 400 g, 4 °C. Subsequently, cells were resuspended in sterile L-15 (Leibovitz) medium containing 10% heat-inactivated fetal calf serum (FCS, Gibco) and antibiotics (penicillin– streptomycin, Gibco). Cells were counted in a Neubauer chamber and adjusted to a concentration of 5x10\(^6\) cells ml\(^{-1}\) in L-15 medium. Six hundred microliters of the cellular suspensions were cultured at 15°C and exposed to 4 and 20 field-collected microlitter particles, representing the “Low” and “High” conditions respectively, with the lowest microlitter concentration being in line with future modelled estimates (Isobe et al., 2019). Sterility was ensured in all microliter and cell preparation phases by working under laminar flow cabinet.

Cells were treated for 2 and 72 hours at 15°C with gentle rotary shaking to ensure a continuous contact with microlitter particles. Because the bottom trawl net was not equipped with any temperature sensor, the incubation temperature was selected according to the near real-time numerical model MEDSEA\_ANALYSISFORECAST\_PHY\_006\_013 (Clementi et al., 2021), resolving for variable “sea_water_potential_temperature_at_sea_floor (bottomT)” using sampling location, date and depth as input. A negative control consisting of cells incubated at same conditions without microlitter was tested. Three technical replicates per biological sample were used in all experimental groups. Intracellular ATP value, as a proxy of cell viability/cytotoxicity (Schoonen et al., 2005), was then quantitatively evaluated using the ATPlite assay (PerkinElmer) in 96 well plates following the manufacturer’s instructions: 50 µl of cell lysis and 50 µl substrate solutions were added to 100 µl cell suspensions per replicate and shaken for 5 min. Resulting homogenates were transferred to opaque well plates (OptiPlate-96, PerkinElmer) and luminescence was measured using a microplate reader (Wallac Victor2, PerkinElmer), following a 10-minute dark adaptation period.

h. Validation of microlitter as a carrier of biological agents

Following a 72-hour incubation of primary cultures with microlitter following an alike experimental design as above, 10 µl of cell suspension from each experimental group was qualitatively observed under a Zeiss microscope equipped with a colour 8 video camera.
(AxioCam MRC) and a software package (KS 300 and AxioVision). Multiple sets of photographs at random frames were taken per each experimental group and total counts of microorganisms were quantified over a 100,000 µm² area by an operator unaware of treatments.

i. Data analysis, visualization and statistics

Stomach content particle abundance was tested for statistical significance between species using an independent samples t-test with the null hypothesis of equal population means between groups. Datasets were checked for normality and homoscedasticity. A log-transformation was applied to meet the normal distribution assumption.

The relation among species and ingestion of microlitter particle types by color was examined with a chi-square test on a two-way contingency table. The null hypothesis assumed no association between variables. Results are reported as $\chi^2_{df}=test$ statistic.

Cytotoxicity data, grouped by species, time, organ and treatment, were tested for statistical significance using a one-way ANOVA with the null hypothesis of equal population means among groups, followed by a Tukey’s HSD post-hoc test in case the main effect of the models was significant. Datasets were checked for normality and homoscedasticity.

Microbial count was analyzed with the rank-based nonparametric Kruskal-Wallis test because datasets, many of which were zero-inflated, did not meet the assumptions for parametric testing. The null hypothesis was that samples were drawn from the same population or from populations whose medians did not differ.

A comparison of microlitter densities retrieved from representative scientific literature was visualized as mean±SD items m⁻³ with the R “forestplot” package v2.0.1. Data reported in other units than items m⁻³ was excluded from the analysis. Studies were organized hierarchically by location and year, and box size was set to constant.

j. Ethics statement

Ethical review and approval was not required for this study because animals were sampled from the marine environment in strict compliance of the provisions of Directive 2010/63/EU on
the protection of animals used for scientific purposes, and were not subject to any experimental manipulation.
3. Results

a. Qualitative and quantitative characterization of microlitter in the water column

Anthropogenic marine litter mainly of plastic origin and in the form of filaments, fragments and films was found in all water samples taken in the Civitavecchia area, i.e. SUP1-SUP4 (Fig. 2, Table 1). Both microlitter (250 μm < x < 5 mm) and macrolitter (5 mm < x < 6 cm) categories were identified.

Fig. 2
SUP4 and SUP2 had the highest and lowest microlitter particle densities with 0.35 and 0.23 items m$^{-3}$, respectively (Table 1). SUP4 and SUP 1 had the highest and lowest macrolitter particle densities with 0.023 and 0.008 items m$^{-3}$, respectively (Table 1). Their average abundance among all samples was $0.30 \pm 0.02$ items m$^{-3}$ and $0.014 \pm 0.003$ items m$^{-3}$ (Fig. 3A).

All following data refer to the microlitter fraction. With regards to particle shape, filaments, fragments and films were observed. In particular, the highest and lowest concentration of filaments were found in SUP4 (0.12 items m$^{-3}$) and SUP3 (0.02 items m$^{-3}$), respectively; the highest and lowest concentration of fragments were found in SUP3 (0.17 items m$^{-3}$) and SUP1 (0.039 items m$^{-3}$), and the highest and lowest concentration of films was found in SUP4 (0.18 items m$^{-3}$) and SUP2 (0.07 items m$^{-3}$) (Fig. 3B and Table S1).

The vast majority (51%) of filaments belonged to size class C and a smaller population (5%) to size class A (5%). Fragments fell for the most part within size class B (38%), while size class E was the least represented (3%). Films mostly belonged to size classes B and C (39%), while size class E was found in the 1% of cases (Fig. 3C and Table S2).
Filaments from all tows were mostly white (38%) and black (37%); less frequent colors in terms of abundance were blue (23%) and yellow (1%). Fragments were more chromatically diversified: while most of them were white (34%), blue and green fragments were found with a percentage of 24% and 21%, respectively; red (7%), black (3%) and yellow (2%) fragments were less abundant. Films were mostly blue (60%), followed by white (17%) and green (15%); yellow (2%) and red (2%) films were less frequent (Fig. 3D-F and Table S1).

b. Characterization of quantity, quality and chemical composition of microlitter in fish stomach content

Microlitter particles were found in 100% *Merluccius merluccius* specimens and in 5 out of 6 (83.3%) *Mullus barbatus* (Fig. 4).

A higher abundance of microlitter was found in the stomach contents of hake (14.67 ± 4.10 items) than mullet (5.50 ± 1.97 items), but the difference between group means was not statistically significant (t₁₀=2.045, p=0.068) (Fig. 5A).
A

$t_{10} = 2.045, p = 0.068$

- **M. merluccius**
- **M. barbatus**

B

Cumulative microlitter abundance

M. merluccius
M. barbatus

C

Microlitter size classes (%)

Filaments
Fragments

M. merluccius
M. barbatus

D

Filaments

- 2.63%
- 11.84%
- 18.42%
- 5.26%
- 53.95%
- 7.89%

E

Filaments

- 33.33%
- 25.00%
- 25.00%
- 8.33%
- 8.33%

F

Filaments

- 18.18%
- 4.55%
- 18.18%
- 4.55%
- 54.55%

G

Filaments

- 66.67%
- 16.67%
- 16.67%
The retrieved microlitter was classified as filaments and fragments, and no films were found. Filaments were the most abundant shape type, with 76/88 particles (83.36%) in hake and 22/28 (78.57%) in mullet, respectively (Fig. 5B).

*M. merluccius* ingested mostly filaments and fragments in the B and A size ranges, respectively. The most represented filament size class in *M. barbatus* stomach contents was B, while fragments were equally assigned to the three size classes (Fig. 5C).

Filaments found in the stomach contents of *M. merluccius* were generally blue (53.95%) and black (18.42%) followed by red, green, white and other colored types (all below 12%). Blue, black and green filaments were found in similar percentages also in *M. barbatus* (54.55%, 18.18% and 4.55%, respectively). Microlitter fragments in hake and mullet were mostly white (33% and 66.67%, respectively) and red (25% and 16.67%, respectively); however, green, blue and other colored-fragments were retrieved only from hake, while black fragments were only found in mullet (Fig. 5D-G). Differences in microlitter stomach content by color between species was not statistically significant either for filaments ($X^2(5)=9.38$, $p=0.094$) or fragments ($X^2(5)=5.63$, $p=0.34$).

A representative subsample of item types found within stomach contents of both species was analyzed by μFT-IR. Particles of plastic origin, namely polypropylene, HDPE and polyamide (87%, 72% and 58% match with corresponding reference spectra, respectively), were identified (Fig. 6 A-C).
To evaluate the presence of microorganisms and consequently validate the role of microlitter as carrier of biological agents, 10 μl cell suspension from all cultures following a 72-hour incubation were qualitatively assessed by optical microscopy. Numerous bacilliform bacteria (grey arrowheads), unicellular fungi (white arrowheads) and flagellates (black arrowheads) were observed in microlitter-exposed samples (Fig. 7 B-D) but not in the controls (Fig. 7 A).
Their abundance was quantified over a 37.000 µm² area and normalized to 100.000 µm² area per species, organ and microlitter concentration. A variable degree of biological contamination was found in conditioned primary cultures of both species (Table 2).

**Table 2**

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<th>Organ</th>
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<th>high</th>
<th>ctrl</th>
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<th>95% CI microbial count [L;U]</th>
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For the hake, a significant effect of microlitter concentration on microbial counts was found only in HK primary cultures (H statistics=6.16, p=0.025). For the mullet, statistical significance was evidenced for microbial counts in G and HK (H statistics=7.62, p=0.036).

**d. Microlitter cytotoxicity in *M. merluccius* and *M. barbatus* cells**

Microlitter cytotoxicity was evaluated based on the viability of cells from G, HK and SPL following a 2- and a 72-hour long incubation with two environment-sampled microlitter concentrations (Fig. 8).
Exposure to microlitter did not induce any statistically significant decrease in intracellular ATP following the short incubation time in either species or organ (Table S3a-b).

In *M. merluccius*, microlitter induced a decrease in median cell viability after a 72-hour incubation in primary G cultures at the high concentration and in both HK and SPL cultures at the low concentration. The linear model fit to splenic cell culture data following the 72-hour exposure explained the 62.3% of ATP levels variation, even though differences among experimental groups were slightly non-significant (*p*=0.054) (Table S3a).

In *M. barbatus* primary cultures from all organs, the median intracellular ATP levels in the Low and High groups were lower than those of respective controls. Such a decrease revealed a
statistically significant main effect of microlitter concentration on splenic cells ($F(2,6) = 10.8, p = 0.01$), with the overall treatment effect explaining almost 80% of ATP levels variation of the model ($\eta^2 = 0.783$) (Table S3b). Pairwise comparisons between control and the “Low” and “High” groups were statistically significant ($p$ adjusted = 0.0113 and 0.027, respectively) (Fig. 8).
4. Discussion

In this work we applied a multidisciplinary approach combining oceanographical, spectroscopical, cellular and microscopical methods to characterize the quality and quantity of microlitter particles in the coastal epipelagic water column and in the digestive tract of two commercially-valuable Mediterranean fish species; we also preliminarily addressed the cytotoxic potential of field-collected microlitter on primary cultures of cells extracted from mucosal (gills) and lymphoid (head kidney and spleen) organs.

The marine litter causes multiple environmental, economic, social, political and cultural impacts (Barboza et al., 2019; Galgani et al., 2019; GESAMP, 2015; UNEP, 2014), especially to the health and functioning of organisms and ecosystems (Corinaldesi et al., 2021; Garcia-Vazquez et al., 2018; Rios et al., 2007). At the European level, such pollutant was included among the 11 qualitative descriptors of the Marine Strategy Framework Directive upon which the quality of the marine environment is assessed (European Parliament, 2008/56/EC). Since 2004, when the term microplastic was coined, extensive research has demonstrated the ubiquity of plastic pollution in several matrices such as beaches (Fortibuoni et al., 2021; Prevenios et al., 2018), sediments (Piazzolla et al., 2020; Renzi et al., 2018) and seawater (Atwood et al., 2019; Capriotti et al., 2021) - regardless of how remote they are (Cincinelli et al., 2017; Lusher et al., 2015). Microlitter was retrieved from all water samples taken within the framework of the PISCES project in a much higher (~20-fold) average concentration (0.30 ± 0.02) than litter particles > 5 mm (0.014 ± 0.003 items/m³). Keeping in mind the environmental and biological severity of litter < 5 mm, our results are in good agreement with microlitter concentrations reported from other areas of the Mediterranean Sea, Yellow Sea and oceanic waters (Baini et al., 2018; Cincinelli et al., 2017; Collignon et al., 2012; Constant et al., 2018; Cózar et al., 2015; de Lucia et al., 2018, 2014; Expósito et al., 2021; Fagiano et al., 2022; Fossi et al., 2012, 2016; Kazour et al., 2019; Lusher et al., 2015; Panti et al., 2015; van der Hal et al., 2017; Zhang et al., 2017; Zhao et al., 2014) (Fig. 9), suggesting that even surveys that are not extensive in either duration or sample sizes can reliably capture the extent
of microlitter pollution. This is auspicable to minimize the impact of research-related anthropogenic activities. An exception was represented by the Eastern Mediterranean Sea, which appears to be much more polluted than the western basin. We must highlight that data dispersion could not be quantified from Cózar et al. (2015) and Constant et al. (2018) as only as mean items m$^{-3}$ were reported, and that data from Vasilopoulou et al. (2021) was discarded because of non-informative results (41.31±112.05 mean±SD items m$^{-3}$ - SD could be back-calculated from standard error because a sample size was clearly indicated by authors).

The relationship between the marine biota and microlitter was so far mostly evaluated by ingestion rates (e.g. Rios-Fuster et al., 2019; Savoca et al., 2019). Here we confirmed that both our target species ingest plastic materials that range in size from 250 to 3000 μm and are represented by polypropylene, high density polyethylene and polyamide items (Chabuka and Kalivas, 2020; Primpke et al., 2018). Benthic macrolitter in the area was recently described quasi-quantitatively, and the most abundant categories were attributed to plastic (Mancini et al., 2021). We call the attention on the fact that the same chemistry was also demonstrated for items sampled from marine sediments (Piazzolla et al., 2020) and in the

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### Fig. 9
atmosphere (Lucci et al., 2021) of the same area, pointing to the high and pervasive dispersion of anthropogenic litter in multiple environmental compartments.

MPs are thought to be mistaken for or even purposefully chosen instead of food (Clark et al., 2016; Ling et al., 2017) probably also depending on their color (Du et al., 2021; Wright et al., 2013). The presence of both shape types in the stomach of *M. merluccius* and *M. barbatus* and the lack of statistically significant differences based on the chromatic factor support the idea that microlitter may be ingested non-selectively by these two species, even though a biomagnification origin cannot be excluded. Hake and mullet were chosen as experimental models for a variety of reasons: on one hand they are among the most targeted demersal fish species by the Mediterranean deep-sea fisheries and the two most fished target species in the shallow area of the coastal sector (Sabatella et al., 2017; Tiralongo et al., 2021); on the other, they are regarded as bioindicators in coastal marine ecosystems and display a benthic feeding behavior in part of (juvenile *M. merluccius*) or throughout their lifespan (*M. barbatus*) (Carrozzi et al., 2019; Esposito et al., 2014). Moreover, some authors already described the occurrence of MPs in these two species (Atamanalp et al., 2021; Avio et al., 2015; Bellas et al., 2016; Digka et al., 2018; Giani et al., 2019; Mancuso et al., 2019) and MPs were demonstrated to abound in superficial sediments in the study area (Piazzolla et al., 2020).

A lower number of studies aimed at also elucidating physiological impacts exposed fish to pristine commercially-available MPs under controlled laboratory conditions. Their bioavailability was demonstrated and effects such as altered feeding behaviour, metabolic disorders, energy depletion, growth impairment, delayed development, compromised immune response, reproduction and lifespan were reported (Botterell et al., 2019; Espinosa et al., 2019, 2017; Guerrera et al., 2021; Mazrulis et al., 2015; Rios-Fuster et al., 2021; Sendra et al., 2021; Yong et al., 2020).

Recently, beach-sampled microlitter was employed in *in vivo* experiments on the European sea bass *Dicentrachus labrax* (Zitouni et al., 2021) and medaka *Oryzias latipes* (Pannetier et
al., 2020) to investigate survival, development, uptake, oxidative stress and genotoxicity following the administration of a microlitter-spiked feed. Their results showed the ability of environmental microplastics to i) accumulate in fish organs, ii) significantly affect the activity of enzymes involved in the antioxidant defense system and iii) induce DNA damages following acute exposures. HK primary cultures were also employed to define the impacts of non-environmental MPs on the abundance and antibody response of B lineage cells in rainbow trout (Zwollo et al., 2021): a lower rate of B cell development together with reduced expression of Ig heavy chain genes were found, suggesting that not only innate but also adaptive immunity may be threatened by MPs.

Despite some similarities with the three just-mentioned studies may be perceived, we must highlight that no other research had ever investigated the apical cytotoxic event in primary cell cultures derived from select fish mucosal and lymphoid organs following their exposure to microlitter that had been collected in the same water column from where animals originated (search conducted on Web of Science on October 24, 2021). We believe that our results, obtained in an attempt to bridge the fields of biological oceanography and experimental toxicology, are biologically significant because i) microlitter particles and fish specimens originated from the same sampling site, ii) microlitter cytotoxicity was measured by the well-established and unambiguous direct luciferase-based quantification of cellular ATP (Cree and Andreotti, 1997; Mahto et al., 2010) iii) primary cultures were obtained from organs that are critical for ensuring immune barrier and competency and iv) the strategy suitability for testing for MP toxicity was overall demonstrated and recently reviewed in details (Revel et al., 2021).

In addition, fish have been increasingly established as experimental models in the fields of biomedical sciences and toxicology because they share many similarities with higher vertebrates immunology-wise (Miccoli et al., 2021; Scapigliati et al., 2018).

It was known that MPs are a carrier of biological agents (Amaral-Zettler, 2019; Kiessling et al., 2015), and our data confirm this. Because a dedicated experiment aimed at molecular taxonomy could not be set up due to limited microlitter availability, flagellates were attempted
a classification on phenotypic properties. Based on flagellar features and because they are extremely common in marine plankton, where they can be found free-swimming or attached to bacterial mats or other surfaces, we suggest that flagellates belong to *Paraphysomonas* sp., *Spumella* sp. or aloricate Bicosoecida. The fate of MPs in the water column and sediments can be influenced by microbes (Rogers et al., 2020). Once the microlitter is ingested, its associated microorganisms may colonize the gastrointestinal tract of the host, possibly affecting its welfare: in fact, harmful microorganisms, including potential human and animal pathogens, were found associated to litter (Zettler et al., 2013) and, according to Zwollo et al. (2021), serious consequences may arise due to the reduced ability to respond adequately to pathogens because of suboptimal humoral immune responses.

Taking into account cytotoxicity data (Fig. 8) and the lack of statistically different microbial counts observed in *M. barbatus* spleen cultures (Table 2), splenic cell subpopulations appeared to be the most sensitive to microlitter exposures among all investigated organs. No further reduction in ATP levels were seen in the High compared to the Low condition, suggesting that such a pollutant can impact cell viability also at low concentrations that are in line with modelled estimates over the next three decades. These results are concerning because spleen, together with thymus and kidney, is the major lymphoid organ of teleosts where adaptive immune responses are generated (Flajnik, 2018; Zapata et al., 2006). It is important to highlight that neither the physiological endpoints reported in the large majority of scientific literature nor our results herein presented provide insight into the molecular mechanisms underlying microlitter toxicity pathways; rather, they inform about apical events manifested either by the whole organism or primary cell cultures, respectively. However, the novelty of our approach was to provide data on a lower, possibly more predictive, level of biological organization (cellular vs. organismal) by means of so-called New Approach Methodologies, which heavily rely on *in vitro* testing. This is compliant with the 3Rs principle in animal testing in addition to having been validated by the latest internationally-agreed test
guidelines (OECD, 2021) and supported by regulatory toxicology roadmaps (e.g. EPA’s strategic vision).

5. Conclusion

In conclusion, the present study has characterized the anthropogenic litter in the coastal epipelagic Northern Tyrrhenian Sea and the digestive tract of commercially-relevant fish species, validated the microliter fraction as a carrier of biological agents and, for the first time, demonstrated that splenic cell viability is negatively affected following exposure to such a contaminant. Future investigations with larger sample sizes, cell cultures from additional organs, either primary or continuous, and more in-depth methodological approaches are warranted for clarifying possible differences in susceptibility of the biota to anthropogenic microliter.
6. Author contribution

AM: Conceptualization, Funding acquisition, Data curation, Formal analysis, Visualization, Supervision, Project administration, Writing - original draft, Writing - review.

EM: Conceptualization, Funding acquisition, Investigation, Writing - Review & Editing.

PRS: Methodology, Investigation, Writing - original draft, Writing - Review & Editing.

GDV: Methodology, Resources, Writing - Review & Editing.

GS: Resources, Supervision, Writing - Review & Editing.

SP: Supervision, Writing - Review & Editing

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