MONITORING MICRO-LITTER INGESTION IN MARINE FISH:

A HARMONIZED PROTOCOL FOR **MSFD** & **RSCs** AREAS

VERSION 01

INDICIT II
INDICATOR • IMPACT • TAXA

CONTENTS

CONTRIBUTORS TO THE PROTOCOL
Matiddi M., Pham C.K., Anastasopoulou A., Andresmaa E., Avio C.G., Bianchi J., Chaieb O., Palazzo L., Darmon G., de Lucia G.A., Deudero S., Sozbilen, D., Eriksson J., Fischer E., Gómez M., Herrera A., Hattia E., Kaberi H., Kaska Y., Kühn S., Lips I., Miaud C., Gambaiani, D. Nelms S., Piermarini R., Regoli F., Sbrana A., Setälä O., Settiti, S., Soederberg L., Tomás J., Tsangaris C., Vale M., Valente T., Silvestri C. 2021

1.0 INTRODUCTION

The Marine Strategy Framework Directive (2008/56/EC) with the new Commission Decision (2017/848/EU) replaced the INDICATOR 10.2.1 "Trends in the amount and composition of litter ingested by marine animals (e.g. stomach analysis)" (2010/477/EU), with the CRITERION D10C3 "The amount of litter and micro-litter ingested by marine animals is at a level that does not adversely affect the health of the species concerned".

This implies that also ingested micro-litter *(artificial polymers and other materials)* should be monitored. Two bio-indicator species have already been chosen for macro-litter: the seabird Fulmarus glacialis for the Northern European sea waters *(van Franeker, 2004; van Franeker* **et al.***, 2011)*, and the loggerhead sea turtle Caretta caretta for the Mediterranean basin (Matiddi *et al.*, 2011; 2017; 2019). Otherwise, no one of the many candidate species has yet been chosen to monitor micro-litter ingestion.

Expert researchers of the MSFD TG-ML (2013) elaborated several basic requirements for biota, which can be considered for the purposes of monitoring and selecting target species, such as:

- **Sample availability:** "Samples of a monitoring species should be available with adequate numbers of individuals over a wider span of time and space".
- **Regular litter consumption:** "Frequency of occurrence and amounts of plastic found in stomachs should be high enough to allow detection of trends over time and geographical patterns".
- **Marine feeding habits:** "Stomach contents should reflect only the marine environment".

Fish seem to be the most suitable organisms to be used as bio-indicator of micro-litter ingestion but, up to now, not one of the proposed species have been selected (UNEP/MAP WG.439/Inf.12.2017; Fossi *et al.*, 2018; Bray *et al.*, 2019). Moreover, the different methods and procedures of sampling and analysis are still not harmonized (Silvestri *et al.*, 2018). Recently, a growing body of literature on plastic ingestion by fish have been produced showing that plastic ingestion occurs in many species, not only in laboratory experiments but also in the field including commercial species. The heterogeneity of applied methods for the collection and analysis of samples, does not allow a reliable comparison of results.

The present protocol comprises the INDICIT II EU project Deliverable, which considers the results of previous EU Projects (INDICT; BASEMAN; PLASTOX; MEDSEALITTER; PLASTICBUSTER) and scientific literature on this topic. A dedicated questionnaire was sent to researchers, institutes and delegates of the MSFD TG ML across the different EU countries. A pilot action to test the protocol is in progress by: ISPRA (Italy), FRCT (Portugal), CNR-IAS (Italy); EPHE (France); INSTM (Tunisia); HCMR (Greece); EOMAR-ULPGC (Spain); PAU DEKAMER (Turkey); UniMa (Italy).

2.0 SAMPLING METHOD

2.1 Fishing Gears

The sampling method represents a potential source of bias that must be considered when developing a harmonized protocol. Depending on the target species, trawling net seems to be the most reliable way of sampling, including opportunistic approaches (fish stock assessments cruises, including demersal and pelagic fish stocks assessment, and stomach content analysis performed on regular basis). A comparison of four types of nets to verify the incidence of plastic ingestion during sampling was investigated by Davison and Asch *(2011)*. Results showed that the percentage of fish ingesting plastics did not significantly differ amongst the different tested nets. Nevertheless, regurgitation of ingested material or net feeding can normally occur when fish are caught. Using polymer nets, traps and ropes can result in a bias. Reducing as much as possible the exposure of the animals to the fishing gear could result in minimizing contamination. Thus, sampling duration should be kept as short as practically realistic. Long hauls should be avoided: as the net is scraped on the seabed and re-suspends microplastic particles. Moreover, macroplastics are continuously captured and fragmented during the haul, potentially resulted in a cloud of fishes and plastic in the cod end of the net *(Silvestri et al., 2018)*. Gill nets should be chosen for sampling in shallow waters and hot spots (harbor, river mouth, etc.) or MPAs. However, other methods are also permitted while ensuring that all sources of bias are reduced to the minimum. Hook should be avoided due to reduction of random samples (hungry animal) and bait's secondary contamination.

2.2 Collection of Samples

Samples should be collected directly on board, checking the fish for any disease and ensuring that all fish showing signs of net feeding or regurgitation are rejected (check in the mouth). Avoid bias due to the regurgitation of plastic items caused by the expansion of the swimbladder. Samples collected at the fish market are not allowed.

It is important to highlight that time of haul and rapid changes in depth could increase stomach regurgitation (Fig. 1). In some species (e.g. Gadidae) regurgitation is a very common phenomenon and increases with sampling depth. Visually undetectable regurgitation was difficult to document, but also occurs and results in negative bias in stomach content estimates *(Kühn and Franeker ,2020; Valente et al., 2020).*

Therefore, it is recommended to reject all fish with everted stomach or completely empty stomach *(Lusher et al., 2017).* All individuals should be rinsed with ultrapure water and frozen upon collection.

Fig.1 On the right an everted fish stomach, not suitable for analysis; on the left a normal one *(source: Valente T., ISPRA).*

To minimize spatio-temporal variability, all samples should be collected from the same location and at the same time (ideally from the same haul). However, there is some flexibility to collect the samples, during the same week or month, as long as they are collected in the same season. Some boreal fish species do not forage during winter times; therefore, this season should be avoided *(Kühn et al., 2020),* at least in the North Sea.
It could be possible to collect samples coming from ongoing monitoring programs, such as fish stock assessments cruises (e. g. MEDITS, SOLEMON, ICES-DATRAS, etc.). EU Data Collection Framework (DCF) surveys could be used as a platform to conduct the sampling of the target species. A cost-effective sampling strategy could be to sample the selected fish species already used for age determination. In this case it should be considered that most DCF surveys are conducted 1-2 times per year in a fixed time period and in a standardized way, with a station grid covering the main target area. This allows to have the same sampling design for different Countries.

2.3 Proposed Target Species

Nowadays we are still far to officialize a target species for all the MSFD marine waters. A broad and harmonized monitoring strategy based on comparable data is needed. Here are some considerations based on previous experiences and recent studies.

Target species should be selected according to some characteristics. In particular, they should:

- be representative of specific environmental compartments;
- have a commercial value;
- have a wide distribution in the MSFD (and RSCs) areas;
- already be described as regular litter consumers by different research studies.

Moreover, it is fundamental to underline that different feeding behaviors and habitat uses entail the necessity to select different fish species to investigate all the marine compartments within a specific area.

Many target species have been proposed for Mediterranean Sea *(UNEP/MAP WG.439/Inf.12.2017; Fossi et al., 2018; Bray et al., 2019)*, and also in deep-water habitat (*Alomar and Deudero, 2017; Valente et al., 2019),* Atlantic Ocean (*Herrera et al., 2019)* and North Sea *(Kühn et al., 2020).* However, only few studies found the influence of fish biological parameters on micro-litter ingestion rate *(Compa et al., 2018; Sbrana et al., 2020).*

Species widely distributed in most of MSFD areas (i.e., Baltic Sea, North Sea, Celtic Sea, Bay of Biscay, Mediterranean Sea and Black Sea) – and therefore suitable as bioindicator species, are Anchovy *(Engraulis encrasicolus)*, Sprat *(Sprattus sprattus)*, Atlantic horse mackerel *(Trachurus trachurus)* and Atlantic mackerel *(Scomber scombrus)*. Unfortunately, all these species are pelagic species. Therefore, other benthic/demersal target species must be chosen at Regional level.

The bogue *(Boops boops)* is a suitable demersal species, which has been widely investigated during the MEDSEALITTER project (Garcia-Garin *et al.*, 2019; Sbrana *et al.*, 2020; Tsangaris *et al.*, 2020). Although this species occurs in the North Atlantic and in the Mediterranean Sea, it is not present in the North Sea/Baltic Sea areas. Other widely distributed benthic/demersal species are European hake (Merluccius merluccius - Eastern Atlantic from Norway and Iceland to Mauritania, Mediterranean Sea and southern coasts of the Black Sea; Froese and Pauly, 2019) and Red mullet (Mullus barbatus – Eastern Atlantic from British Isles to Canary Islands, Mediterranean and Black Seas; Hureu, 1986; Froese and Pauly, 2019).

The species within the genus *Mullus* (benthic-feeder), *Merluccius* (demersal) and *Scomber* (pelagic-feeder) have been chosen for the pilot action in INDICIT II EU project.

Among those, the genus *Merluccius* already has shown to have different critical aspects since it:

- presents a high degree of regurgitation in samples from bottom trawling;
- has a wide length range, which affects its feeding behavior;
- is of very high commercial value in some countries, and therefore too expensive;
- in the Mediterranean, the species has been considered as Vulnerable according to IUCN Red list *(Di Natale et al., 2011);*

2.4 Sample size

Thirty samples seem to be a feasible number of fish able to combine right effort and statistical analysis. If the frequency of occurrence of ingested microplastics is 5% in a population, there is 95% chance to detect them with a sample of 30 fish (Di Giacomo and Koepsell, 1986).However, for very clean areas it could be necessary to increase the number of fish to 50 individuals.

The INDICIT consortium fixed the minimum sample size at 30 individuals for each species from each sampling site. Considering that 3 habitat compartments should be assessed (benthic, demersal, pelagic) for each monitored site, a total of 90 individuals per site should be collected (90 fish=3 species x 30 specimens /site).

The number of sampling sites should be planned according to the spatial scale of the investigated area (GSA; Sub-Region; Local hot spots; MPAs; etc.).

2.5 Fish size

In order to reduce possible variability in microlitter ingestion due to the variation of feeding behaviors of fish during life stages (e.g. juveniles/adults), it is suggested to choose comparable individuals. In the INDICIT pilot action the common length for target species have been fixed around the size at first maturity, according to the FishBase dataset *(Froese and Pauly, 2019).*

In this case the sizes measured as the total length (Fig. 2) (i.e. from the tip of the snout to the tip of the longer lobe of the caudal fin) for the chosen target species, including a variability of 10% were fixed as follow:

- *Merluccius merluccius* 30±4.5 cm
- *Scomber scombrus* 25±3 cm
- *Mullus barbatus* 12±2 cm

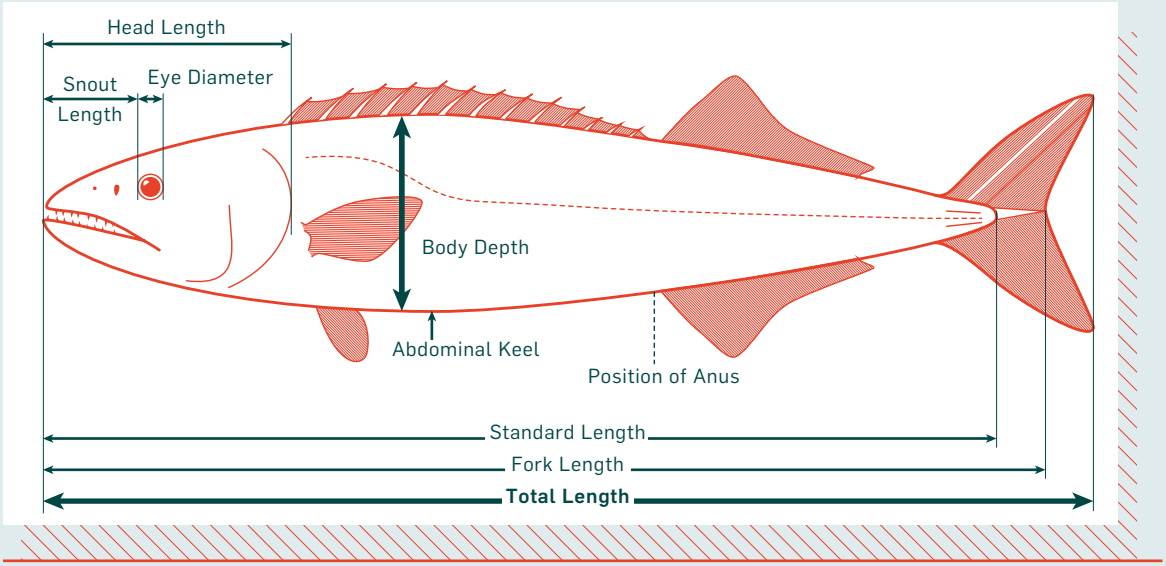

Fig.2 Total length *(source: Fishbase.org)*

It could be possible that the size of first maturity for a single species changes in different areas, but the same life stage should be chosen for a comparable data.

3.0 LAB ACTIVITIES

Frias and Nash (2019) reported that the term *'microplastics'* coined by Thompson *et al.* (2004), is used to describe the smaller plastic particles recorded. However, there is still no all-inclusive definition that accurately encompasses all criteria that could potentially describe what a microplastic is. Arthur *et al.* (2009) proposed to fix the upper limit to five millimeters, but up to now there is not an established lower limit and all the researchers in their studies use the common terminology *"plastic particles smaller than 5 mm".*

The Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection defines microplastics as "plastic particles < 5 mm in diameter, which include particles in the nano-size range (1 nm)" (GESAMP, 2016). This requires to measures the diameter instead of length of micro-items and it means that a thin filament longer than 5 millimeters should be counted. Moreover, Galgani *et al.*, (2019) define micro-litter as particles that pass through a 5 mm mesh screen but are retained by a lower one, according to the chosen size class.

Following this consideration the definition of Microplastics used in this document is: ***"All sorts of small particles of plastic, less than 5 mm in two of the three dimension or diameter that pass through a 5 mm mesh screen but are retained by a lower one, according to the chosen size class"*** and the definition of Microlitter ***"All sorts of small manmade particles, less than 5 mm in two of the three dimension or diameter, that pass trough a 5 mm mesh screen but are retained by a lower one".***

Nowadays, nano-size particles (*i.e.* smaller than 1 nm) are extremely challenging to identify for most stakeholders. It is too time consuming, require sophisticated and expensive equipment and is not easily applied for many samples. Therefore, at this stage, it is not recommended for monitoring purposes.

Choosing different microplastic lower size limits can lead to a different representation of the incidence of marine microplastic ingestion by fish. It seems obvious that the frequency of occurrence of ingested microplastic will increase as the lower limit of detection decrease.

For example, Rummel *et al.* (2016) counted particles above 500 nm and the percentage of fish with microplastic ingested was 5.5%. Güven *et al.* (2017) used a mesh size of 26 nm and the percentage of fish with ingested microplastic was 58%. Although crucial, someone follow to use length at the longest point (Lusher *et al.*, 2013), while for most studies the lower size limits of the investigated plastics is not specified.

This implies that a lower size limit must be fixed for monitoring, in order to have comparable data.

3.1 Size Class Reporting

There are different size class proposals in literature (Box 1).

Box 1. Size class proposals for microlitter

- **EMODNET:**
  20 nm<x<200µm
  200<x<300µm
  300µm<x<1mm
  1mm<x<5mm
- **Frias and Nash (2019):**
  1 µm<x<100µm
  100<x<350µm
  350µm<x<5mm
- **Eriksen *et al.*, (2014):**
  330µm<x<1mm
  1mm<x<5mm
- **BASEMAN project (Frias *et al.*, 2018):**
  lower limit of microplastic monitoring in sediment 100 µm
- **GESAMP (2016):**
  1 nm-5mm
- **Valente *et al.* (2019):**
  100 µm<x<330µm
  330 µm<x<1mm
  1mm<x<5mm

According to the INDICIT consortium, the size classes proposed by Valente *et al.* (2019) are considered the most suitable for monitoring purpose.

In fact, the lower limit is harmonized with BASEMAN proposal (Frias *et al.*, 2018) for monitoring microplastic in sediments (100 µm), and the size classes from 330µm up to 5mm (330 µm<x<1mm; 1mm<x<5mm) are comparable with data coming from microplastic sea surface monitoring, by using Manta trawl (MSFD TG-ML 2013).

Detection of smaller items lower of 100 µm in size should be taken into consideration only for research studies.

Size Class 1: 1mm<x<5mm;

Size Class 2: 330µm<x<1mm;

Size Class 3: 100µm<x<330µm.

Table 1. Proposed size class for monitoring (from Valente *et al.*, 2019)

3.2 Analytical Analysis

Several methods and protocols were applied to assess microplastic ingestion by fish (Lusher *et al.*, 2017). The most accurate procedures involve the digestion of the entire gastrointestinal tract with its content (Bianchi *et al.*, 2020) and in general it happens using potassium hydroxide (KOH) or hydrogen peroxide (H_2O_2).
It is important to consider that both reagents at high concentration and high incubation temperatures can affect the polymers structure/integrity (Karami *et al.*, 2017; Avio *et al.*, 2015) and that fish food preference may determine the best suitable digestion protocol (Bianchi *et al.*, 2020).

Considering accuracy, cost/benefit and time consume, only two methods are shown below for monitoring purpose, although other procedures might be equally appropriate.

The use of enzymes or other methods to degrade bio-organic materials are not reported due to the high costs and the procedure complexity.

3.3 Airborne Contamination

Synthetic fibers are ubiquitous and biological laboratories are not well equipped to completely avoid such secondary source of contamination.

Precautions are essential during all steps of the sample processing and different procedures are available to reduce or evaluate sample contamination.

Airborne secondary contamination and cross contamination must be avoided or reduced as much as possible and kept under control using blank samples.

According to the MSFD TG-ML (Galgani *et al.*, 2013), secondary contamination cannot exceed 10% of the results. Recently, Avio *et al.* (2020) proposed that if the blank is contaminated, microlitter items with similar characteristics (shape, color, polymer type, size) should be excluded from the results (i.e. the specific microlitter type found in the blank control, should be subtracted from the same specific microlitter type value in the samples of the same batch).

Box 2 reports general guidelines to ensure limited levels of contamination.

Box 2. Guidelines to reduce airborne contamination

The following guidelines are useful to ensure limited levels of contamination:

1. Close the window and reduce personnel in the laboratory;
2. During the procedure of dissection and filtration, samples must be processed under a laminar flow cabinet or glove box (Torre *et al.*, 2016);
3. During stereo-microscopy observation of the membrane, Petri dishes must be covered by a glass dish;
4. Dress only cotton wear and coats;
5. Use only glass and metal labware, whenever possible;
6. Clean all the equipment with ultrapure Milli-Q water before each sample analysis;
7. Perform a blank control at every step;
8. Place a damp filter paper in a petri dish in the working area to assess any airborne contamination.

3.4 Dissection Procedure

1. Rinse the outside of the fish with ultrapure Milli-Q water before dissection.

2. Clean all the equipment with ultrapure water.

3. Each fish should be weighed (g up to the first decimal) and the total length measured (up to the nearest mm).

4. Fish must be dissected in laboratory to extract the entire gastrointestinal tract (GI), from the mouth to the cloacae.

5. Reject all fish showing signs of regurgitation (stomach protruded from the mouth).

6. Fish with completely empty stomach should be excluded to ensure that fish that had regurgitated are not included.

7. Entire GI tracts must be weighed (grams up to the first decimal), before digestion.

8. Optional: Stomach and intestine should be weighed and analyzed separately.

9. Optional: Weigh the liver and the gonads (grams up to the first decimal).

10. Optional: Record the sex of the animal.

11. Put the GI in a glass beaker covering the top with paper foil or aluminum or glass.

12. Digest all the GI including the wall using hydrogen peroxide (H_2O_2 15%) or potassium hydroxide (KOH 10%).

Using H_2O_2 (MEDSEALITTER project modified):

i. Add gradually in the Beaker 20 ml of hydrogen peroxide ($H_2O_2$15%) for each gram of GI (in 2 aliquots if GI ≤2 g or more aliquots for GI ≥2 g).

ii. Optional: add HNO_3 up to 5% to increase tissue degradation (Bianchi *et al.*, 2020).

iii. Incubate samples on hot plate or hot bath or oven (≤ 40°C) adding supplementary H_2O_2 15% when evaporate, until all organic matter is digested (see below).

iv. Add 100 ml of H_2O Milli-Q and stir it using a magnetic stirrer.

v. Use a blank sample to test for possible ambient contamination (add similar volume of 15% H_2O_2 as that used in the samples in a beaker without samples and follow the protocol.

Using KOH (Rochman *et al.*, 2015 modified):

i. Add in the Beaker potassium hydroxide KOH (10% w/v, 3× tissue volume).

ii. Optional: Incubate samples on hot plate or hot bath or oven (≤40 °C) to increase digestion speed.

iii. Optional: It is suggested to neutralize the

→

3.4 *Continued*

digestate before filtration, adding 1 M citric acid solution (Thiele *et al.*, 2019).

iv. Use a blank sample to test for possible ambient contamination (add similar volume of KOH 10% as that used in the samples in a beaker without samples and follow the protocol).

It should be noted that both reagents could affect polymers structures and colors. For this reason, maintain temperature bath at no more than 40 °C and digestion bath for no more than 5 days. Reduce temperature and time of exposure, according to the organic digestion rate.

13. In order to standardize data, pre-filter the solution through 100 µm sieve, under laminar flow cabinet, collecting all the material by washing the sieve with ultrapure Milli-Q water.

14. Filter the material retained by the sieve, on a fiber glass membrane or anodisc or other membrane, with a mesh size less than 100 µm, using vacuum pump.

15. Rinse glass funnel above the membrane with ultrapure water.

16. Insert the membrane on a Petri dish, covered by glass top.

17. Place the Petri dish in a clean cupboard for drying membrane at room temperature.

18. Detect number and position of fibers on the membrane, using stereomicroscope, before opening the dish. Note position of the particles that should be checked.

19. Detect all the microliter items under stereomicroscope. Uncertain microplastic can be recognized using optical microscope or hot needle or Spectroscopy.

20. Categorize the collected items according to both size classes and shape

21. At least 10% of the collected items should be analyzed using micro-spectroscopy FT-IR or Raman (MSFD TG ML, 2013).

According to the micro-spectroscope used, procedures from 11 to 19 can change.

Box 3. Digestion steps using H_2O_2

Digestion steps according to the MEDSEALITTER proj. mod.:

- Add gradually in the Beaker 20 ml of hydrogen peroxide ($H_2O_2$15%) for each gram of GI (in 2 aliquots if GI ≤2 g or more aliquots for GI ≥2 g).
- Optional: add HNO3 up to 5% to increase tissue degradation (Bianchi *et al.*, 2020).
- Incubate samples on hot plate or hot bath or oven (≤ 40 °C) adding supplementary H_2O_2 15% when evaporate, until all organic matter is digested (see below).
- Add 100 ml of H_2O Milli-Q and stir it using a magnetic stirrer.
- Use a blank sample to test for possible ambient contamination (add similar volume of H_2O_2 15% as that used in the samples in a beaker without samples and follow the protocol.

Box 4. Digestion steps using KOH

Digestion steps according to Rochman *et al.* (2015) mod.:

- Add in the Beaker potassium hydroxide KOH (10% w/v, 3× tissue volume).
- Optional: Incubate samples on hot plate or hot bath or oven (≤40 °C) to increase digestion speed.
- Optional: It is suggested to neutralize the digestate before filtration, adding 1 M citric acid solution (Thiele *et al.*, 2019).
- Use a blank sample to test for possible ambient contamination (add similar volume of KOH 10% as that used in the samples in a beaker without samples and follow the protocol).

3.5 Use of Spectroscope

Fibers are ubiquitous and generally correspond to the 70-90% in number of items of the collected marine micro-litter but, they are not always composed by synthetic material. According to the new Commission Decision (2017/848/EU), ingested micro-litter must be categorized as artificial polymer and others. Avio *et al.* (2020) reported that more than 80% of the ingested fibers in 500 marine organisms of the Adriatic Sea, were made of natural or semi-synthetic origin (e.g. rayon, cotton, silk, cellulose, wool). This new study makes mandatory the use of spectroscopy to sub-divide polymeric fibers from other.

Fibers should be reported separately to non-fibrous plastics to allow comparison with other studies.

Fibers are only from textile and should be noted in a separate category from filaments (e.g. fishing line).

4.0 DATA REPORTING

A specific template for data collection is proposed in Annex 1 with basic and optional information required.

It is divided in two sheets, which specify information on fish (sheet 1) and on items (sheet 2):

- Sheet 1 (for fish): Species; Data on the origin of the sample (country, location, date, latitude, longitude, gear); Fish biometric parameters (total length, total wet weight – record if fresh or defreeze, liver weight, gonads weight, sex, stomach weight, intestine weight, gastrointestinal weight); occurrence of micro-litter items in the gastrointestinal tract; total number of micro-litter items in the gastrointestinal tract; total number of micro-litter items for each category).

- Sheet 2 (for items): Species; Category; Size; Size class; Color; Opacity; Polymer identity.

Considering items categories, here it is reported the Kovac Viršek *et al.* (2016) proposal for monitoring micro-litter on the sea surface, modified including the differentiation between fiber and filament (Annex 2):

- Fiber: micro-particle from textile. They are the most abundant type of micro-litter particles. They can be short or long, with different thicknesses and colors;

- Filament: threadlike artificial polymer element. It is elongated, thin and less flexible than a fiber;

- Film: layer, foil. They appear in irregular shapes; Compared with fragments, they are thinner and more flexible;

- Fragment: rigid thick, with sharp crooked edges and an irregular shape. They can be in a variety of different colors.

- Granule: spherical shape, in comparison with pellets, they have a regular round shape and usually a smaller size, around 1 mm in diameter. They appear in natural colors (white, beige, brown).

- Pellet: only from industrial origin, they are usually flat on one side and can be of various colors, irregular, round shapes, and normally bigger in size, around 5 mm in diameter.

- Foam: They most often come from large particles of styrofoam. They are a soft, irregular shape.

Other information required are:

- Total number of fish;

- Total FO% (Frequency of Occurrence= Fish with ingested Plastic/Total samples);

- Total number of items for size class;

- Total number of items for shape category (Annex 2);

- Total number of items for color: white (include yellow); black (include brown); green (all the tonalities); blue (from sky blue to light blue); red (including orange and pink); other (including multi-colors); each one as transparent or opaque.

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Annex 1.

Template for Data Collection*

ID (XX_Yy_zzz)	Species	Country	Location	Date (dd/mm/yyyy)	Latitude	Longitude	Fishing Gear	length (cm)	weight (g)	liver (g)	gonads (g)	GI (g)	Occurence	Items (N)	Fibers (N)	Filaments (N)	Fragments (N)	Granules (N)	Pellets (N)	Films (N)
IT_Sc_001	*Scomber colias*	Italy	Anzio	07/08/2019	41,211617	12,696367	net	26,8	150,5	1,2	0,1	5,8	1	3	0	1	2	0	0	0
IT_Sc_002	*Scomber colias*	Italy	Anzio	07/08/2019	41,211617	12,696367	net	28,1	167,5	1	0,8	7,1	1	2	0	1	1	0	0	0
IT_Sc_003	*Scomber colias*	Italy	Anzio	07/08/2019	41,211617	12,696367	net	27	140,7	1,1	0,1	5,7	1	1	0	1	0	0	0	0
IT_Sc_004	*Scomber colias*	Italy	Anzio	07/08/2019	41,211617	12,696367	net	27,3	158,1	0,7	0,6	5,6	0	0	0	0	0	0	0	0
IT_Sc_005	*Scomber colias*	Italy	Anzio	07/08/2019	41,211617	12,696367	net	27,3	152,2	1,2	1,7	6,6	0	0	0	0	0	0	0	0
IT_Mb_001	*Mullus barbatus*	Italy	Anzio	09/08/2019	41,545867	12,221433	trawl	23,4	162,4	0,1	0,5	9,3	0	0	0	0	0	0	0	0
IT_Mb_002	*Mullus barbatus*	Italy	Anzio	09/08/2019	41,545867	12,221433	trawl	24,2	192,9	0,1	0,3	6,2	1	5	0	1	4	0	0	0
IT_Mb_003	*Mullus barbatus*	Italy	Anzio	09/08/2019	41,545867	12,221433	trawl	24,1	182,7	0,1	0,0	8,3	0	0	0	0	0	0	0	0
IT_Mb_004	*Mullus barbatus*	Italy	Anzio	09/08/2019	41,545867	12,221433	trawl	24,5	187,0	0,2	0,2	8,4	0	0	0	0	0	0	0	0
IT_Mb_005	*Mullus barbatus*	Italy	Anzio	09/08/2019	41,545867	12,221433	trawl	24,6	169,8	0,2	0,3	5,4	1	2	0	1	1	0	0	0
IT_Mb_006	*Mullus barbatus*	Italy	Anzio	09/08/2019	41,545867	12,221433	trawl	21	117,1	0,1	0,2	3,1	0	0	0	0	0	0	0	0

Excel sheet 1 keys (for fish)

- **ID:** Sample identification code. It must be unique, reporting at least information on the origin country and the species. The suggested format is XX_Yy_zzz, where: XX = country initials; Yy = acronym of the species; zzz = progressive number. More complex structures are allowed, as long as they are specified;
- **species:** Binomial name of the species (Genus species).
- **country/location/date/latitude/longitude/gear:** Data on the origin of the sample.
- **length/weight/liver**/gonads**/sex**/stomach**/intestine**/GI:** Fish biometric parameters, namely: length = Total length; weight = total wet weight (record if fresh or defreeze); liver = liver wet weight; gonads = gonads wet weight; sex = male/female/not determined; stomach = full stomach wet weight; intestine = full intestine wet weight; GI = full gastrointestinal wet weight (stomach + intestine). Total length must be reported to the nearest mm; Weight measures must be reported to the nearest 0.1 g.
- **occurrence:** absence/presence (0/1) data on the occurrence of micro-litter in the gastrointestinal tract.
- **items:** total number of micro-litter items in the gastrointestinal tract.
- **fibers/filaments/fragments/granules/pellets/films/foams:** total number of micro-litter items for each category. Keys for determining categories are provided in Annex 4.

* Reported data are fictitious. **Optional information

Annex 1. *Continued*

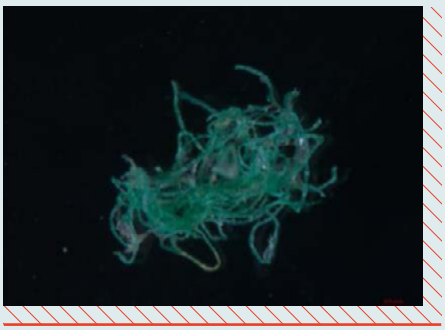
ID (XX_Yy_zzz)	Species (Genus species)	Organ (S/I)	ID (XX_Yy_zzz(w))	Count	Species (Genus species)	Size (µm)	Size Class (1/2/3)	Color	Opacity (T/O)	Polymer
IT_Sc_001	*Scomber colias*	S	IT_Sc_001(1)	1	filament	1625	1	blue	T	nylon
IT_Sc_001	*Scomber colias*	S	IT_Sc_001(2)	2	fragment	847	2	black	O	polypropylene
IT_Sc_001	*Scomber colias*	I	IT_Sc_001(3)	3	fragment	849	2	black	O	polypropylene
IT_Sc_002	*Scomber colias*	I	IT_Sc_002(1)	1	filament	2077	1	blue	O	nylon
IT_Sc_002	*Scomber colias*	I	IT_Sc_002(2)	2	fragment	1075	1	blue	O	polystyrene
IT_Sc_003	*Scomber colias*	S	IT_Sc_003(1)	1	filament	666	2	red	T	polyethylene
IT_Mb_002	*Mullus barbatus*	S	IT_Mb_002(1)	1	filament	655	2	blue	T	polypropylene
IT_Mb_002	*Mullus barbatus*	S	IT_Mb_002(2)	2	fragment	157	3	blue	T	polyethylene
IT_Mb_002	*Mullus barbatus*	I	IT_Mb_002(3)	3	fragment	629	2	blue	T	polyethylene
IT_Mb_002	*Mullus barbatus*	I	IT_Mb_002(4)	4	fragment	138	3	green	O	polyethylene terephthlate
IT_Mb_002	*Mullus barbatus*	I	IT_Mb_002(5)	5	fragment	256	3	red	O	polyvinylchloride
IT_Mb_005	*Mullus barbatus*	I	IT_Mb_005(1)	1	filament	184	3	blue	T	polypropylene
IT_Mb_005	*Mullus barbatus*	I	IT_Mb_005(2)	2	fragment	425	2	blue	O	polypropylene

Excel sheet 2 keys (for items)

- **ID:** Sample identification code (see excel sheet 1 for details).
- **species:** Binomial name of the species (Genus species).
- **organ**:** Stomach/Intestine; tract of the digestive system in which the item was found.
- **ML:** Micro-litter item identification code. It must be unique, reporting ID and a progressive number which identifies the item.
- **count:** cumulative number of items found in a sample.
- **category:** micro-litter category. Keys for determining categories are provided in Annex 2.
- **size**:** particle diameter.
- **size class:** 1) 1 mm – 5 mm; 2) 330 µm – 1 mm; 3) 100 µm – 330 µm.
- **color:** particle color.
- **opacity:** Transparent/Opaque.
- **polymer:** Polymer identity ascertained through spectroscopy.

* Reported data are fictitious. **Optional information

Annex 2.

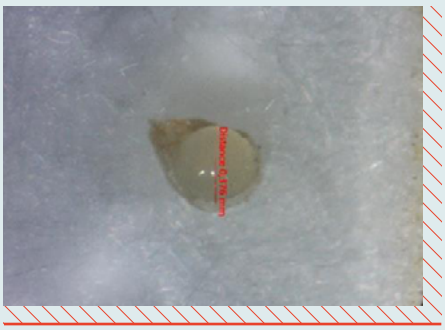


A. Fiber

b. Filament

c. Film

d. Fragment



e. Granule

f. Pellet

g. Foam

Micro-litter Categories:

a. **Fiber:** micro-particle from textiles, often made of non-totally synthetic material. Fibers can have different length, thickness and color;
b. **Filament:** threadlike, elongated and thin artificial polymer element. Usually less flexible than a fiber;
c. **Film:** layer, foil irregular in shape, thinner and more flexible than a fragment;
d. **Fragment:** rigid thick, with sharp crooked edges and an irregular shape. They can be in a variety of different colors;
e. **Granule:** spherical shape, regular round shape. Around 1 mm in diameter, they appear in natural colors (e. g. white, beige, brown);
f. **Pellet:** only from industrial origin. They are usually flat on one side and can be of various colors. Irregular, round shapes, normally around 5 mm in diameter;
g. **Foam:** soft, irregular shape. They most often come from large particles of Styrofoam.