DeFishGear

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PROTOCOL FOR MACRO LITTER INGESTED IN FISH STOMACHS

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February, 2015

1 Protocol for macro litter ingested in fish stomachs

1.1 Introduction

Studies on the ingestion of marine litter by fish are sporadic, rare and usually constitute infrequent events as part of diet studies. If a large amount of marine litter is found in stomachs of a particular species, it could be treated as part of a diet study. For this reason a detailed diet study protocol is given below. However, if someone wants to focus only on ingested marine litter it is not necessary to identify all prey organisms ingested.

1.2 Fish diet/litter study methodology

1.2.1 Sampling

1. Fish can be sampled in three ways with priorities as specified:
   1 – fish sampling by DEFISHGEAR team
   2 – fish sampling in collaboration with fishermen (possibly with WP6 connection)
   3 – fish sampling done on the fish market
2. The location where the fish were captured must be known in all cases.
3. A sample size of at least **30**individuals per speciesis recommended.
4. Three different species will be used. Fish species are recommended on the list bellow:
   Demersal
   - *Soleasolea*
   - *Mullussurmuletus/ Mullusbarbatus*

In the category of demersal fishes you can choose one species of Mullus sp. If you can not get Mullus sp., than you can use Soleasolea (like in Slovenia).

Mesopelagic
- *Pagelluserythrinus*

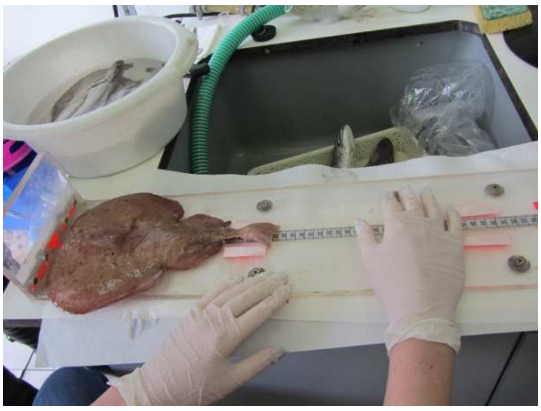
Pelagic
- *Sardinapilchardus*

5. The samples are frozen immediately after capture and are transported to the laboratory.Record the following information:
   a. fishing location
   b. date of capture
   c. sampling gear
   d. day time
   e. depth

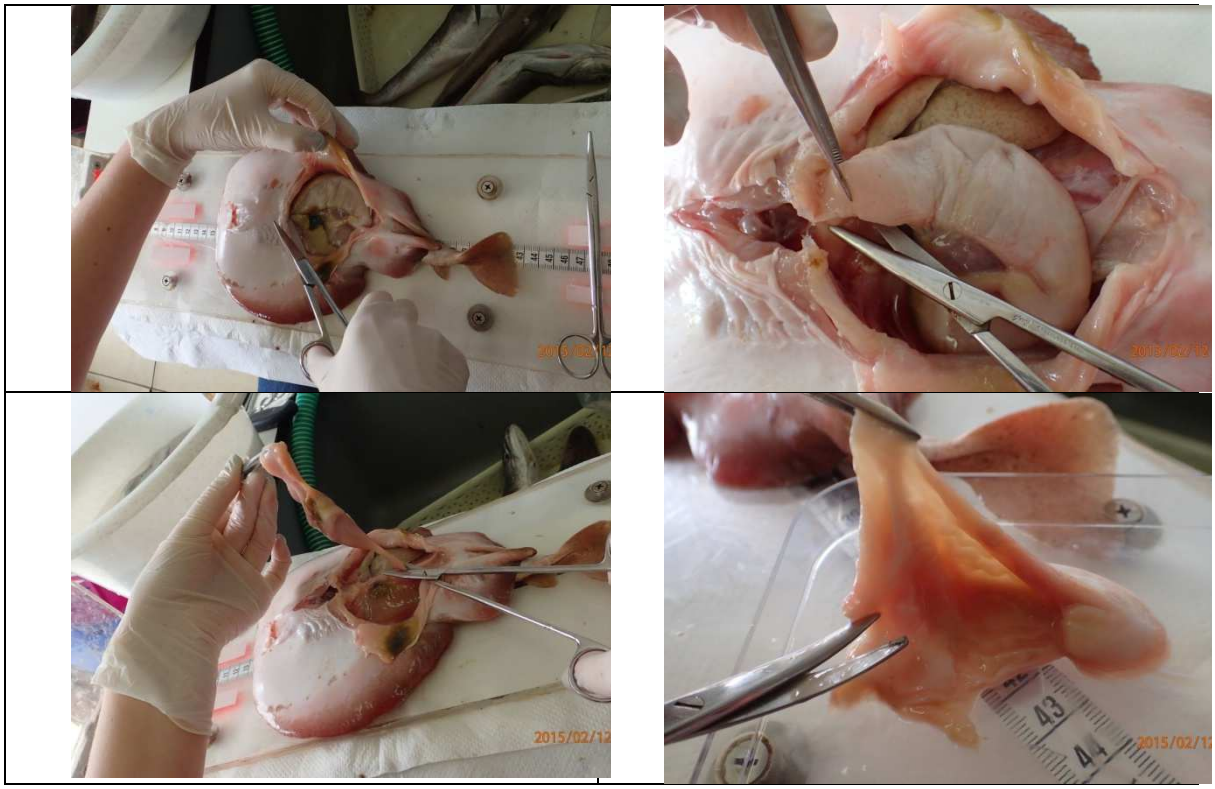
1.2.2 In the Laboratory

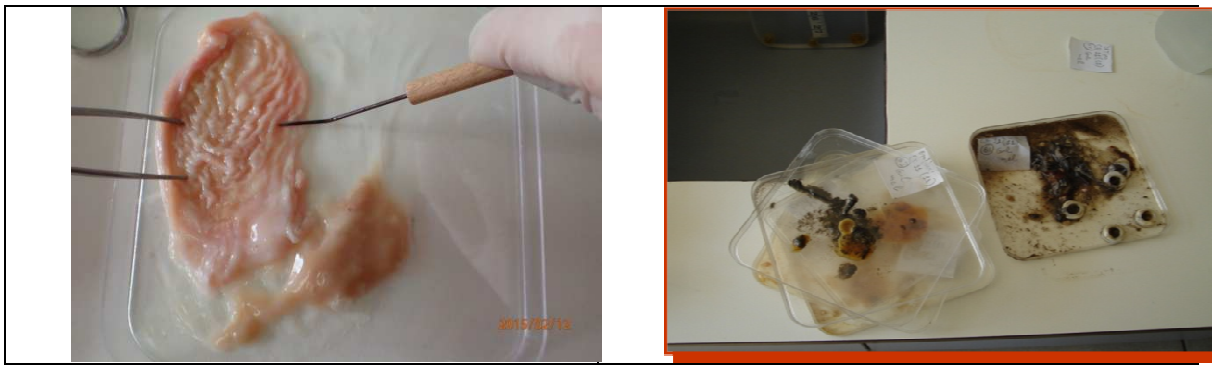
1. Give an ID to each individual

2. Record the following biological parameters for each individual:
   a. Length (mandatory)
   b. Weight (mandatory)
   c. Sex
   d. Maturity stage



3. Fish will be dissected carefully to avoid cutting internal organs. Remove stomach and intestine and keep their content separately into petri-dishes.Be careful to annotate the fish ID in each petri-dish.





4. Stomach and intestine contents are weighted separately. Sorting under a binocular stereoscope. Prey or litteritems will be sorted into separate categories (P_i) and their number and weight will be recorded per category.

Identify the prey items to the lowest possible taxonomic level. Keep the unknown items separately and count and weight them too. Some preys present several identification problems, especially when the prey is almost digested. Sometimes you can find only otoliths from fish or beaks from cephalopods or fragments from crustacean. If these items are characteristic of a certain taxonomic group you can identify it.

Separate the litter items, identify the category they belong, count and weight them. Take measurements of the litter item size. Litter categories should be classified according to Master list of categories of litter proposed by MSFD TSG10 Report.

When it is not possible to analyse at once all stomach and intestine contents, you can keep them frozen and analyze them at a later day.

5. Data entry in an excel file or a data base.

1.3 Diet analysis

Several indices are used to describe the fish diet. The most common of them arepresented below.

1. The vacuity index (VI) = [(number of empty stomachs or intestines/ number of stomachs or intestines examined) x 100] was estimated (Hyslop, 1980).
2. Stomach or intestine fullness can be calculated in two different ways:
   (i) The fullness index expressed as the percentage of six categories of an empirical scale from 0 to 5 with corresponding to 0 empty and 5 to very full stomachs/intestines)
   (ii) the repletion index (RI)= [(StW or IntW)/ NW * 100] (Morato et al., 2000), StW= stomach content weight, IntW= intestine content weight, NW= net weight.
3. The percentage frequency of occurrence (%F) = the number of stomachs/intestines containing a given prey item/ total number of non-empty stomachs or intestines examined x 100.
4. The percentage numerical abundance (%N) = the number of prey items of a given prey category in all non- empty stomachs or intestines / total number of prey items in all stomachs/ intestines x 100.
5. The percentage weight (% *W*) = the weight of prey items of a given prey category in all non-empty stomachs or intestines / total number of food items in all stomachs or intestines x 100.
6. The Index of Relative Importance (IRI%) as modified by Hacunda (1981) [IRI = (% N + % W) x % F] and expressed as percentage.
7. Other more specific or statistical analyses depend on the data and the study.

1.4 Litter analysis

Some of the above mentioned indices can also be used for litter analysis as follows:

1. The percentage frequency of occurrence (%F) = the number of stomachs/intestines containing a given litter item/ total number of non-empty stomachs or intestines examined x 100.
2. The percentage numerical abundance (%N) = the number of litter items of a given litter category in all non- empty stomachs or intestines / total number of litter items of all categories in all stomachs/ intestines x 100.
3. The percentage weight (% *W*) = the weight of litter items of a given litter category in all non- empty stomachs or intestines / total number of litter items of all categories in all stomachs or intestines x 100.

In addition, in order to examine what part of the total stomach or intestine content constituted the ingested litter, the following index can also be used:

4. The percentage (%) of litter weight (all litter items from all categories) / total stomach or intestine content weight.

Annex 1.

Published papers in the Mediterranean

The negative impacts of marine litter (e.g. ingestion,entanglement) on marine organisms (such as turtles, seabirds,marine mammals, fish and invertebrates) have been recognizedmainly during the last few decades. However, information for the ingestion of marine debris by organisms (turtles, seabirds, marine mammals, fish) list sporadic, rare or infrequent as part of diet studies. Especially for the Mediterranean, few publications are available and most of them are presented below:

l. Anastasopoulou, A., Mytilineou, C., Smith, C.J., Papadopoulou, K.N., 2013. Plasticdebris ingested by deep-water fish of the Ionian Sea (Eastern Mediterranean).Deep Sea Res. Part I 74, 11–13.

2. Galgani, F., Hanke, G., Werner, S., Oosterbaan, L., Nilsson, P., et al.2013. Guidance on Monitoring of Marine Litter in European Seas. MSFDTechnical Subgroup on Marine Litter (TSG-ML).http://publications.jrc.ec.europa.eu/repository/bitstream/JRC83985/lb-na-26113-en-n.pdf

3.Anastasopoulou, A., Smith, C.J., Mytilineou, Ch., Papadopoulou, K.N., 2013. Anthropogenic litter on the sea bottom and ingested by fish in the deep waters of the Eastern Ionian Sea. Annex III, p. 25, SCMEE Report of SAC FAO. FAO HQ, Rome, Italy, 18-20 February 2013.

4. Camedda A., Marra S., Matiddi M., Massaro G.,CoppaS., Perilli A., Ruiu A., Briguglio P., Andrea de Lucia G., 2014. Interaction between loggerhead sea turtles (*Carettacaretta*) and marine litter in Sardinia (Western Mediterranean Sea). Marine Environmental Research 100, 25-32.

5.Codina-García M., Militão T., Moreno J., González-Solís J., 2013. Plastic debris in Mediterranean seabirds. Marine Pollution Bulletin 77, 220–226.

6. Lazar B., Gracan R., 2011. Ingestion of marine debris by loggerhead sea turtles, *Carettacaretta*, in the Adriatic Sea. Marine Pollution Bulletin 62, 43–47.

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MONITORING MICROPLASTIC LITTER IN BIOTA

Protocol for biota sampling and sample separation

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June, 2015

2 Biota sampling and microplasticseparation - protocol

2.1 Introduction

Microplastics comprise a very heterogeneous assemblage of pieces that vary in size, shape, colour, specific density, polymer type, and other characteristics. For meaningful comparisons, it is important to define methodological criteria to quantify metrics like the abundance, distribution and composition of microplastics and to ensure sampling effort is sufficient to detect the effects of interest. The aim of this protocol is to maximise consistency and comparability of data collection in the framework of the DeFishGear project. In DefishGear project, fishes and mussels are the proposed indicator organisms for microplastics analysis in biota.

2.2 Equipment needed

2.2.1 Sampling equipment

- GPS
- Cooler containers (ice boxes)
- Aluminum foil or aluminum trays for fish packaging
- Latex gloves without powder

2.2.2 Sample separation equipment

- Measuring ruler
- Analytical Balance
- Dissection scissors
- Scalpels and blades
- Dissection forceps
- Pinpoint tweezers
- Petri dishes
- Filter paper
- Stereomicroscope (min. 80x zoom; recommended also: transmission light with dark field, polarization contrast and ring light)
- Glass flasks or beakers
- H_2O_2 30%
- Hot plate / bainmarie / oil bath
- Filtered or distilled water
- Fume hood
- Büchner funnel or a vacuum filtering device
- 2µmmembrane filters (e.g. Nucleopore Track-Etch Membrane Whatman)
- Glass vials
- Permanent marker

Optional:

- Concentrated saline solution (250 g NaCl/L H_2O)
- Magnetic stirrer
- Glass pipettes (e.g. 10ml)
- FT-IR spectrometer

- Lab coat
- Latex gloves without powder
- Glass filter paper (e.g. Millipore, **APFD04700**, Type 4, retention 2.7 µm, circles size 4.7 cm or Millipore, **APFD02500**, Millipore® glass-fiber filters, Type 4, retention 2.7 µm, circles size 2.5 cm)

2.3 Biota sampling protocol

2.3.1 General conditions

- The same fish samples will be used for macrolitter and microplasticsanalysis
- The criteria agreed for selecting the fish species for macrolitter and microplastics analysis in DEFISHGEAR are
  - species should be present in all countries,
  - species should be commercially available fishes,
  - species must be abundant,
  - same animals can be used for macro and micro litter analysis,
  - species should include demersal, mesopelagic and pelagic fish (3 species in total, 1 species per category)
  - a minimum 20 samples per one species should be collected,
  - location where fish has been caught should be known,
  - sampling activities should be done in June (finished by July 2015).
- To reduce contamination from the nets, fish should be caught in standard mesh nets and a confirmatory step should be included using FT-IR to confirm that fragments from the organisms do not match those of the polymer used in the nets.
- Fish may eject stomach contents during sampling so care must be taken to discard such specimens.
- Avoid the use of plastic tools and containers
- Avoid synthetic clothing (e.g. fleece)

2.3.2 Fish sampling

1. Fish can be sampled in three ways with priorities as specified:
   1 – fish sampling by DEFISHGEAR team
   2 – fish sampling in collaboration with fishermen (possibly with WP6 connection)
   3 – fish sampling done on the fish market
2. The location where the fish were captured must be known in all cases.
3. A sample size of at least 20 specimens per species and age group is recommended.
4. Threedifferent species will be used. Fish species are recommended on the list bellow:
   Demersal
   - *Soleasolea*
   - *Mullussurmuletus/ Mullusbarbatus*

In the category of demersal fishes you can choose one species of Mullus sp. If you can not get Mullus sp., than you can use Soleasolea (like in Slovenia).

Mesopelagic
- *Pagelluserythrinus*

Pelagic
- *Sardinapilchardus*

5. The following parameters should be recorded immediately after sampling:
   - fishing location
   - trawl/fishery type
   - species
   - dateand time of capture
   - depth
6. The fish are frozen immediately after sampling and are transported to the laboratory.

2.4 Microplastic separation from the fish samples

2.4.1 General notes

All materials used for sampling, degradation of organic material and analysis need to be cleaned with filtered distilled water or MilliQ water to prevent contamination. In all steps be careful to avoid contamination with fibers from the air. The usage of covers all the time is proposed.

2.4.2 Laboratory separation of microplastics in fishes

1. Defrost fish samples.
2. Record the following parameters:
   - fish length
   - weight
   - visible deformations and skin condition (e.g. ulcers)
   - gender
   - maturity stage
3. Dissect the stomach and intestine, rinse it with dH_2O, place it in petri dish (marked with a fish ID) and weigh it (in case of small fishes the stomach and intestine should be weighted together; in case of big fishes stomach and intestine should be weighted separately). For large fishes, stomach and intestine contents are removed and placed in glass petri dishes and weighed (stomach and intestine content separately).

Figure 1. Stomach and intestine of fish dissected out.

4. Check the stomach and intestine contents under a stereomicroscope for identification of plastic items. Depending on the size of the organism the entire gut or samples of the gut wall can be examined (e.g. 10cm x 10cm or similar standard area).
5. Remove items of unusual appearance with forceps, categorize it into one of the categories according to categories in the Table 1 and place it on clean filter paper in petri dish.

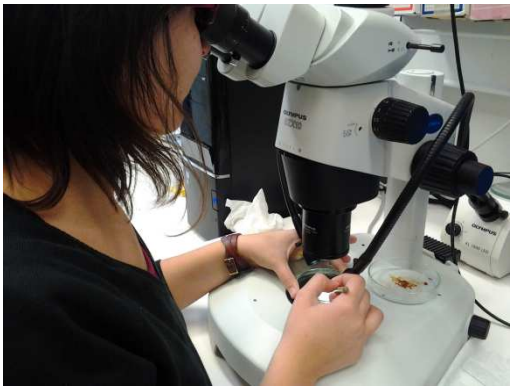
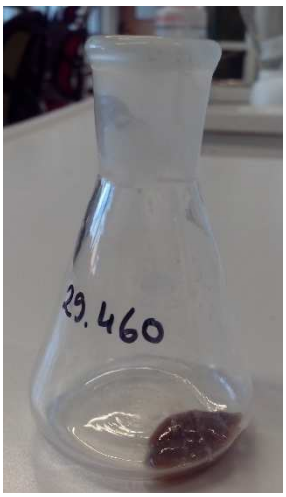


Figure 2. Examination of stomach and intestine under a stereomicroscope.

6. After removing items visualized under stereomicroscope, a digestion procedure using 30% H_2O_2 is suggested to degrade natural organic matter in order to facilitate detection of small microplastic particles.
    a. Transfer the content of stomach and intestine into the conical flask (250 ml with wide neck) (in case of small fishes the stomach and intestine should be weighted and degraded together). (You can use the whole stomach and intestine, not just content, but then the degradation is longer.)



    b. Add 20 ml of 30% H_2O_2 per 1gof wet weight of stomach or intestine or gut.

c. From this step, two different options are possible:

   i. **Separation with flotation:**Incubate at 55 - 65°C on hot plate so long that H_2O_2 evaporate and continue with step **d**(cover with aluminum foil to avoid air contamination). Before you continue with step d make sure that most of the organic matter is removed, if not add H_2O_2 and wait until H_2O_2evaporate.
   ii. **Dilution and filtration:**Incubate for 24h at 55 - 65°C in oil bath or bainmarie and continue with step **e**(cover with aluminum foil to avoid air contamination).

d. Add 100 ml concentrated saline solution (250g NaCl/L dH_2O), stir it at high intensity for 1–2 min using a magnetic stirrer, settle for 1-2 min and transfer

supernatant by a glass pipette onto 0.2 µmembrane filters using a vacuum filtering device. Repeat this step 3 times. Continue with step h.

e. Dilute the content in conical flask with distilled water (recommended MilliQ water) in ratio 1:10.
f. Filter the suspension through the glass fiber filter paper (e.g. Millipore, APFD04700, Type 4, retention 2.7 µm, circles size 4.7 cm).
g. Rinse the conical flask 3 times with 50 ml of distilled water and filter this water. Continue with step h.
h. Dry the filter paper at room temperature through the night(cover e.g. in petri dishes).
i. Check the filter paper for microplastic particles (300 µm – 5 mm) by the use of stereomicroscope.
    i. When finding each microplastic particle, categorize it into one of the categories according to categories in the Table 1 and put it in the Petri dish (or other glass vials), marked with category name. The Petri dish needs to be closed at all times.
    ii. Put Petri dish under the microscope with measuring equipment and measure the size of each particle (measure the longest diagonal), except filaments (If you do not have image analysis program, you do not need to do this, the National Institute of Chemistry Slovenia will do this).
    iii. Weigh the microplastic particles of each category separately in glass vials. Microplastic particles need to be previously dried.
    iv. Post well closed glass vials with microplastic particles (all categories) to the National Institute of Chemistry Slovenia. Please use following address:

National Institute of Chemistry Slovenia
Laboratory for Polymer Chemistry and Technology
dr. Andrej Kržan
Hajdrihova 19
1000 Ljubljana
Slovenia

Note 1: Chemical Institute will do the chemical analysis of particles lying on glass fiber filters. If you have possibility to buy glass fiber filter 2,5 cm in diameter (e.g. Millipore, APFD02500, Millipore® glass-fiber filters, Type 4, retention 2.7 µm, circles size 2.5 cm), we ask you that you send the particles on this filters. Before you put the particles on filters, just make the filters wet with distilled water that particles can adhere on filter. Filters could be post in Petri dishes.

Note 2: In case of uncertainty if item is microplastic or not, collect the item anyway, the final confirmation will be done by chemical analysis. Be careful to not substitute the carapax of crustaceans with plastic. Carapax is more brittle and crumbly.

7. Negative controls and blanks:
    a. In the process of degradation the negative control should be included (the conical flask with 20 ml of 30% H_2O_2 and 180 ml of distilled water is incubated with the other samples and after incubation the solution is filtered and filter paper checked under the stereomicroscope)

b. In the process of separation of microplastic particles under the stereomicroscope the blank sample should be included (the clean filter paper is exposed to air in the working area). Most probably you will have the problems with the contamination with fibers from the air. We suggest that you are very careful with cleaning the laboratory and all used equipment and that you sign down how many fibers were present in blank sample.

2.4.3 Mussels sampling

1. Mussels can be sampled:
   a. in collaboration with fishermen
   b. on the fish market
2. The location where the musselswill be collected must be known in all cases.
3. A sample size of at least 30 - 50 specimens is recommended.
4. The recommended mussel species is *Mytilusgalloprovincialis* / *Mytilusedulis*
5. The following parameters should be recorded immediately after sampling:
   - location of collection
   - habitat (nature or mussel farm)
   - species
   - date and time of capture
   - depth
6. The mussel are frozen immediately after sampling and are transported to the laboratory.

2.4.4 Laboratory separation of microplastics in mussels

1. Defrost mussel samples.
2. Record the following parameters on the mussels selected:
   - mussel length
   - mussel width
3. Dissect the mussel in order to obtain the gills and the hepatopancreas, place it in a conical flask (250ml with wide neck)(marked with a mussel ID) and weigh it (the gills and the hepatopancreas should be weighted together);

Figure 1: Hepatopancreas of mussel dissected out.

4. A digestion procedure using 30%H_2O_2 is suggested to degrade natural organic matter in order to facilitate detection of small microplastic particles.
    a. Add 20 ml of 30% H_2O_2 per 1g of wet weight of gills and hepatopancreas together.
    b. Incubate for 24h at 55 - 65°C in oil bath or bainmarie and continue with step **e**(cover with aluminum foil to avoid air contamination).
    c. Dilute the content in conical flask with distilled water (recommended MilliQ water) in ratio 1:10.
    d. Filter the suspension through the membrane filter (Whatman, Nucleopore Track-Etch Membrane 2 µm).
    e. Rinse the conical flask 3 times with 50 ml of distilled water and filter this water. Continue with step h.
    f. Dry the filter paper at room temperature through the night(cover e.g. in petri dishes).
    g. Check the filter paper for microplastic particles (300 µm – 5 mm) by the use of stereomicroscope.
        i. When finding each microplastic particle, categorize it into one of the categories according to categories in the Table 1 and put it in the Petri dish (or other glass vials), marked with category name. The Petri dish needs to be closed at all times.
        ii. Put Petri dish under the microscope with measuring equipment and measure the size of each particle (measure the longest diagonal), except filaments (If you do not have image analysis program, you do not need to do this, the National Institute of Chemistry Slovenia will do this).
        iii. Weigh the microplastic particles of each category separately. Microplastic particles need to be previously dried (the open weighing dish can be put in desiccator or wait for 24 h to dry the samples on the air, but in closed dish).
        iv. Post well closed glass vials with microplastic particles (all categories) to the National Institute of Chemistry Slovenia. Please use following address:

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Note 2: In case of uncertainty if item is microplastic or not, collect the item anyway, the final confirmation will be done by chemical analysis. Be careful to not substitute the carapax of crustaceans with plastic. Carapax is more brittle and crumbly.

5. Negative controls and blanks:
    a. In the process of degradation the negative control should be included (the conical flask with 20 ml of 30% H_2O_2 and 180 ml of distilled water is incubated with the other samples and after incubation the solution is filtered and filter paper checked under the stereomicroscope)
    b. In the process of separation of microplastic particles under the stereomicroscope the blank sample should be included (the clean filter paper is exposed to air in the working area). Most probably you will have the problems with the contamination with fibers from the air. We suggest that you are very careful with cleaning the laboratory and all used equipment and that you sign down how many fibers were present in blank sample.

How to identify micro litter?

When analyzing sample in search for microplastics, please consider that some particles will be easily visible (colour, shape, size) while others may be trickier to find. Here you can see few suggestions on how to identify microplastics in your sample:

-no cell structure
- uneven, sharp, crooked edges
- uniform thickness
- distinctive colours (blue, green, yellow, etc)

When separating microplastics from your sample be conservative and remove more than less. We can still later on determine real chemical structure of particles. Please consult also photo guide for categories for easier identification of microplastics in the Appendix 2.

Table 1: Categories of micro litter Items taken from EU TG ML Master List[1]. (*New category for uncategorized plastic pieces was added. In this category you can range the plastic items that are not typical for any other category).

Micro litter categories
Fragments (G103, G104, G105, G106)
Pellets (G107, G108, G109, G110, G111)
Granules (G116)
Filaments (G113)
Films (G114)
Foam (G115, G117)
Other (nonplastic materials) (G217)
Uncategorized plastic pieces*

[1] For easier categorization we merged categories G103, G104, G105 and G106 in category of Fragments, categories G107, G108, G109, G110, G111 in category of Pellets, categories G115 and G117 in category Foam.