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Guidance on the Monitoring of Marine Litter in European Seas

An update to improve the harmonised monitoring of marine litter under the Marine Strategy Framework Directive

MSFD Technical Group on Marine Litter

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8 Litter and microlitter ingested by biota and entanglement with litter

8.1 Introduction

This chapter focuses on the criteria reported in the new Commission Decision (Decision (EU) 2017/848) related to the impact of litter on marine biota: D10C3 'The amount of litter and microlitter ingested by marine animals is at a level that does not adversely affect the health of the species concerned' and D10C4 'The number of individuals of each species which are adversely affected due to litter, such as by entanglement, other types of injury or mortality, or health effects'. In the old Commission Decision from 2010 (Decision (EU) 2010/477), there was already the need to develop an indicator that included the amount and composition of litter ingested by marine animals, but there was not a clear link to the health of the animals. As no single species can provide full coverage of all Europe's marine sectors and each marine litter category (from macro to micro), a range of species is needed to monitor marine litter impact.

Given their propensity to ingest litter, their wide distribution and the large range of habitats used during their life, two species are already validated for monitoring ingested litter. The northern fulmar, *Fulmarus glacialis* (Linnaeus, 1761) was chosen as an indicator for the northern European waters (van Franeker et al., 2011), while sea turtles, in particular the loggerhead species *Caretta caretta* (Linnaeus, 1758), were chosen as an indicator for the Mediterranean basin (Matiddi et al., 2011, 2017). For those two species, threshold values have been suggested, while for most other taxa the indicators are still not mature. For assessing the impact of microplastic fish are becoming good candidate bioindicators (UNEP/MAP, 2019; Bray et al., 2019; Matiddi et al., 2021, Valente et al., 2022) even if no individual species has yet been chosen. Moreover, mussels have been investigated by different authors as bioindicator for microplastics (Li et al., 2018; Lusher et al., 2017; Bessa et al., 2019).

Entanglement in marine litter, defined as 'any marine organism wrapped, trapped or stuck in marine litter including fishing gear lost or abandoned' (Silvestri et al., 2021), has been reported to occur worldwide in various species, causing injuries and death, and protocols for monitoring have been developed within the EU projects MEDREGION (Silvestri et al., 2021) and INDICIT II (Loza et al., 2021).

As stated by Decision 2010/477/EU and Decision (EU) 2017/848, knowledge of the impacts of litter on marine life should be improved, especially regarding species affected, impacts on health, standardisation of methods and determination of thresholds. For this purpose, acquiring knowledge on the entanglement of marine organisms in litter for criterion D10C4, the 'impacts of litter on marine life' is also needed. This guidance should be considered for monitoring purposes and the methods described here are thought to apply the necessary degree of accuracy. Other methods are possible but their cost, time and complexity should be evaluated.

8.2 Scope and key questions to be addressed

Existing methods for monitoring marine biota litter ingestion and entanglement that fulfil the requirements of the MSFD are evaluated and reported here in a harmonised way. The methods provided in this chapter can be applied to assess the impact of litter on biota in Regional Seas to ensure comparability of results. The ingestion of marine macro litter and microlitter, and entanglement of marine organisms and the use of plastic litter as nesting material, are considered for inclusion in monitoring guidelines to assess impact. Explanation of different sections building starting from the previous knowledge, are reported in the following bullet points.

- In the North Sea, an indicator is available that expresses the impact of marine litter (the OSPAR ecological quality objective (EcoQO)). It measures ingested litter in northern fulmar and is used to assess temporal trends, regional differences and compliance with a set target for acceptable ecological quality in the North Sea area (van Franeker et al., 2011; OSPAR Commission, 2015a). The combined protocol proposed here can be used for seabirds in general and applied in most north-east-Atlantic countries, where the threshold value was calculated from near-pristine Canadian Arctic data (van Franeker et al., 2021).
- After some consideration and a pilot study conducted by Italian researchers, the experts of the TG ML have chosen the sea turtle *Caretta caretta*, (Linnaeus, 1758) as the target species for monitoring litter ingested by marine organisms in the Mediterranean Sea (Matiddi et al., 2011, 2017; Galgani et al., 2013). The protocol has been improved and made available in several languages (INDICIT consortium, 2018). All stages of manipulations during necropsy and two scenarios for threshold values were reported by Matiddi et al. (2019).

- Protocols for the analysis of marine litter in stranded marine mammals were developed at the International Whaling Commission (IWC) workshop and recently reviewed (IJsseldijkl et al., 2019). The methodology has been harmonised by the Agreement on the Conservation of Cetaceans of the Black Sea, Mediterranean Sea and Contiguous Atlantic Area (ACCOBAMS) and by the Agreement on the Conservation of Small Cetaceans of the Baltic, North-East Atlantic, Irish and North Seas (ASCOBANS).
- The assessment of microlitter ingestion in biota (birds, fish and invertebrates) can be incorporated into the provided protocols even if none of the assessment methods can be considered completely mature at this stage.
- Fish seem to be suitable organisms to be used as bio-indicators of microlitter ingestion and the present protocol comprises the INDICIT II EU project deliverable that considers the results of previous EU projects and scientific literature on this topic (Matiddi et al., 2021). Currently, none of the many candidate fish species have yet been chosen for monitoring microlitter ingestion but many are already investigated and proposed (Bray et al., 2019; Valente et al., 2022;2023).
- A protocol on microlitter ingestion by benthic filter-feeding organisms, such as mussels, oysters, and clams in shallow coastal waters (water depth < 5 m) is proposed.
- Ingestion protocols for invertebrates such as crustaceans, shellfish, worms or zooplankton are not included in this report.
- The monitoring protocols developed to assess the entanglement of megafauna (sea turtles and mammals) and sessile benthic organisms are provided as an easy tool for comparing standardised data and understanding the impact of marine litter on the marine environment, either globally or on a local scale. The proposed protocols are the outputs of the MEDREGION (Silvestri et al., 2021) and INDICIT II (Loza et al.,2021) EU projects.
- In addition, a harmonised protocol for assessing the use of plastic litter as nesting material and associated entanglement mortality in bird breeding colonies, sea turtles and seals is proposed for immediate application.

Key questions are still open and other aspects are crucial issues for further research, and as a result some options are not currently suitable for recommendation for large-scale monitoring applications at this stage. The following points summarise the key open questions that need further development.

- Monitoring of ingestion does not directly reflect a correlation with the health of the species concerned, though this is included in the Commission Decision (EU) 2017/848; only one proxy has been proposed, which is for the loggerhead turtle, where the weight of litter vs food of the gut content is compared (Matiddi et al., 2019).
- The impact of ingested marine litter is most frequently sublethal in effect rather than lethal. Sublethal effects are not easily detected and are difficult to distinguish from impacts resulting from other pollutants. To understand the implication of marine litter ingestion on animal conservation more studies are needed.
- Until now, threshold values have been validated only for norther fulmars (*Fulmarus glacialis*) (van Franeker et al., 2021), and loggerhead turtles (*Caretta caretta*) (Matiddi et al; 2019), while a possible fish GES scenario has been proposed (Matiddi et al., 2021).
- The first definition of microplastic as 'all particles less than 5 mm in diameter' (Arthur et al., 2009) did not define a lowest limit and originated different biases in data comparisons. For MSFD purposes, microlitter is defined as particles of < 5 mm in their maximum length, fixing the lowest limit for monitoring litter in biota as 100 µm.
- To assess the impact of marine litter on both megafauna and benthic organisms by entanglement, it is necessary to quantify the number of individuals of each species that are adversely affected. To do this, the population of a given species present in a specific area and the proportion of entangled animals should be known. Currently, it is not possible to determine this kind of information with certainty, and for this reason an assessment can be made using the frequency of occurrence as a percentage (FO%) of entanglement per region/area and per year (Silvestri et al., 2021).

8.3 Protocol for litter ingestion by seabirds

8.3.1 Protocol name

MSFD protocol for the monitoring of litter ingested by seabirds (Procellariiformes, like fulmars or shearwaters).

8.3.2 Protocol description

The methodology of this protocol follows the OSPAR (EcoQO) methods for monitoring litter items in the stomachs of northern fulmars (*Fulmarus glacialis*). The stomach contents of birds beached or otherwise found dead are used to measure trends and regional differences in marine litter. Background information and the technical requirements are described in detail in documents related to the fulmar EcoQO methodology. A pilot study evaluating methods and potential sources of bias was conducted by van Franeker and Meijboom (2002). Bird dissection procedures, including parameters for age, sex and cause of death, have been specified by van Franeker (2004). Further OSPAR EcoQO details were given by OSPAR Commission (2008, 2010a, 2010b, 2015a, 2015b), van Franeker and the SNS Fulmar Study Group (2011) and van Franeker et al. (2011).

8.3.3 Related marine compartments

Seabirds such as fulmars or shearwaters mostly feed at or near the surface of the sea. Therefore, the water column and especially the water surface are the marine compartment addressed when quantifying litter in the stomachs of fulmars. The plastics in fulmar stomachs mostly consist of mesoplastics (0.5–2.5 cm) and large microplastics (1–5 mm), with a small fraction of macroplastics (> 2.5 cm).

8.3.4 Technical requirements

Bird corpses are stored frozen until analysis. Standardised dissection methods for fulmar corpses have been published in a dedicated manual (van Franeker, 2004) and are internationally calibrated during regular workshops. Stomach content analyses and methods for data processing and presentation of results are described in detail by van Franeker and Meijboom (2002) and were updated in later reports. The methodology has been published in peer-reviewed scientific literature (van Franeker et al., 2011; van Franeker and Law, 2015). For context, some of the methodological information is repeated here in a condensed form.

During dissection, a full series of data is recorded to determine sex, age, breeding status, likely cause of death, origin, and other issues. Age, the only variable found to influence litter quantities in stomach contents, is largely determined on the basis of the development of sexual organs (size and shape) and the presence of the Bursa of Fabricius (a gland-like organ positioned near the end of the gut which is involved in the immune systems of young birds; it is well developed in chicks, but disappears within the first year of life or shortly after). Further details are provided by van Franeker (2004).

After dissection, the stomachs of birds are opened for analysis. Fulmar stomachs have two units: initially, food is stored and starts to digest in a large glandular stomach (the *proventriculus*), after which it passes into a small muscular stomach (the *gizzard*) where the remains of harder prey can be processed through mechanical grinding. To achieving cost-effective monitoring, the contents of the proventriculus and gizzard are combined, but optional separate recordings should be considered where possible.

Stomach contents are carefully rinsed in a sieve with a 1 mm mesh and then transferred to a Petri dish for sorting under a binocular microscope. The 1 mm mesh is used because smaller meshes become easily clogged with mucus from the stomach wall and with food remains. Analyses using smaller meshes were found to be extremely time-consuming and particles smaller than 1 mm are very rare in fulmar stomachs, contributing little to plastic mass. Should the method be applied to other, small species, such as storm petrels or phalaropes, a smaller mesh size may need to be considered.

If oil or chemical pollutants are present, these may be sub-sampled and weighed before rinsing the remainder of the stomach contents. If sticky substances hamper further processing of the litter objects, hot water and detergents can be used to rinse the material clean prior to further sorting and counting under a binocular microscope.

8.3.4.1 Litter categories – source related information

In the fulmar protocol, stomach contents are sorted into the categories shown in Table 8.1, and this categorisation is followed for monitoring marine litter ingestion in seabirds.

Table 8.1. Categories for the classification of items for monitoring marine litter ingestion in biota.

Biota categories for the contents of the digestive tract			
PLA	Plastic	Acronym	All plastic or synthetic items. Note the number of particles and the dry mass for each category
IND	Pellets	ind	Industrial plastic granules (usually cylindrical but oval, spherical or cubical shapes exist)
	Probab ind?	pind	Suspected industrial, used for tiny spheres (glassy, milky, etc.) (i.e. microbeads)
USE	Sheet	she	Remains of sheet from bags, cling-foil, agricultural sheets, rubbish bags, etc.
	Thread	thr	Threadlike materials, pieces of nylon wire, net-fragments, woven clothing, etc.; includes balls of compacted material
	Foam	foam	All foamed plastics, polystyrene foam, foamed soft rubber (as in mattress filling), PUR used in construction, etc.
	Fragments	frag	Fragments, broken pieces of thicker type of plastics; can be a bit flexible but not like sheetlike materials
	Other	Poth	Any other items, including elastics, dense rubber, cigarette filters, balloon pieces, soft air gun bullets and objects. Specific items should be described.
RUB	Other rubbish	Acronym	Any other non-synthetic consumer wastes. Note the number of particles and (in principle) the dry mass for each category
RUB	Paper	pap	Newspaper, packaging and cardboard. Includes multilayered material (e.g. Tetra Pak pieces) and aluminium foil
	Kitchen food	kit	Human food remains (galley waste) such as onions, beans, chicken bones, bacon, seeds of tomatoes, grapes, peppers, melons, etc.
	Other rubbish	rubvar	Other various rubbish, such as processed wood, pieces of metal, metal airgun bullets, lead shot and paint chips. Describe
	Fishhook	hook	Fishing hook remains (not for hooks on which longline victims were caught)
POL	Pollutants (industrial/chemical waste)	Acronym	Other non-synthetic industrial or shipping wastes. Note the number of items and the mass per category (wet mass for paraffin)
POL	Slag/coal	slag	Industrial oven slags (looks like non-natural pumice) or coal remains

	Oil/tar	tar	Lumps of oil or tar (also note as *n* = 1 and *g* = 0.0001 g if other particles are smeared with tar but cannot be sampled separately)
	Paraf/chem	chem	Lumps or soft mush of unclear paraffin, waxlike substances (not stomach oil); if needed, estimate mass by subsampling
	Feather lump	confea	Lump of feathers from excessive preening of fouled feathers (n = 1 with dry mass) (not meaning a few of their own feathers, which is normal)
FOO	Natural food	foo	Various categories, depends on the species studied and the aims of study
NFO	Natural non food	nfo	Anything natural that cannot be considered normal nutritious food for the individual

Source: Adapted from Galgani et al. (2013).

The fulmar categorisation of stomach contents is based on the general 'morphs' of plastics (sheet like, thread like, foamed, fragment, other) or other general rubbish or litter characteristics. This is because particles cannot be unambiguously linked to specific objects in most cases. Where this is possible, in the notes on datasheets, the items should be described and assigned a litter category number using the *Joint List of Litter Categories for Marine Macrolitter Monitoring* developed by the TG ML group (Fleet et al., 2021).

For each litter category/subcategory an assessment is made of the:

- incidence (percentage of investigated stomachs containing litter);
- abundance by number (average number of items per individual);
- abundance by mass (weight in grams, accurate to fourth decimal place per individual).

Due to the potential variations in annual data, it is recommended that 'current levels' be noted as the average for all data from the most recent 5-year period, in which the average is the population average and includes individuals that were found to have zero litter in their stomachs.

As indicated, EcoQO data presentation for northern fulmars is for the combined contents of glandular (proventriculus) and muscular (gizzard) stomachs. The results for all age groups should be combined except for those chicks and fledglings, which should be dealt with separately. Potential bias from age structure in samples should be checked regularly.

8.3.4.2 Size range

In the fulmar monitoring scheme, stomach contents are rinsed over a sieve with a 1 mm mesh prior to further categorisation, counting and weighing. The size of plastics monitored is thus ≥ 1 mm. Unpublished data on particle size details in stomachs of fulmars show that a smaller mesh size would not be useful because smaller items would have passed into the gut.

In the OSPAR Commission fulmar EcoQO approach, the focus is on the mass of each litter category, rather than on the size of individual particles. However, the litter Descriptor of the MSFD makes a distinction between macro litter and microlitter particles, the latter defined as objects where the largest dimension is < 5 mm. Both size groups are common in seabird stomachs. For comparative purposes it is therefore useful to know the proportions of microlitter and macro litter found in seabird stomachs. Whether this assessment of particle size is incorporated into standard monitoring methods or it is evaluated on a more incidental basis will depend on practical and financial considerations. In the current fulmar project, particle size assessment is not standard procedure (particle number and combined mass per litter category only give average size information), but a dedicated study is currently assessing the exact sizes of all particles in a large number of samples from different locations and periods. This dedicated detailed work can be repeated at appropriate time points.

In the seabird studies it is standard to filter stomach contents over a 1 mm sieve, which largely ignores the potential presence of microplastics of < 1 mm in size. In fulmar stomachs, objects of such sizes seem extremely rare, but could potentially be present in gut material in the intestines as a result of the break-up of larger items in the stomach or from secondary (passive) ingestion during zooplankton or fish consumption.

8.3.4.3 Spatial coverage

Dead birds are collected from beaches or from accidental mortalities; they are often long-line victims and fledglings killed on roads, for example (for the methodology, see van Franeker, 2004).

8.3.4.4 Survey frequency

Continuous sampling is required. A sample size of 40 birds or more is recommended for a reliable annual average for a particular area. However, years with low sample sizes can be used in the analysis of trends as the standard trend analyses are based on individual birds and not on annual averages. For reliable conclusions on changes or stability in ingested litter quantities, data over periods of 4–8 years (depending on the category of litter) are needed (van Franeker and Meijboom, 2002). In the OSPAR Commission approach (OSPAR 2015a) recent trends are evaluated over all individuals investigated over the most recent 10 years of data.

8.3.4.5 Maturity of the tool

The method is mature and in use. The OSPAR Commission (2015a, 2015b) has made specific guidelines outlining the requirements of the agreed OSPAR monitoring of plastic ingestion in fulmars in the North Sea. The formal OSPAR requirements use a categorisation of stomach contents that quantifies only the number and mass of the main plastic categories (industrial, user, and their combined total).

8.3.4.6 Regional applicability of the tool

The tool is applicable to the MSFD marine regions where fulmars occur; the Greater North Sea, the English Channel and the Celtic Sea For similar seabird species, including any of the tubenose family, the methodology can follow this protocol. This could, for example, be applied to shearwater species occurring further south in the Atlantic or in the Mediterranean Sea.

8.3.5 Estimation of costs

A cost estimate for fulmar biota monitoring can be based on the current level of funding available for the monitoring project in the Netherlands. This currently amounts to approximately EUR 60000 annually, largely dedicated to personnel costs (based on contract rates by Wageningen University and Research, the Netherlands). This concerns the time invested in coordinating the collection programme by volunteers and other groups (ca. EUR 20000), the lab dissections, stomach analyses and data analysis of approximately 40-50 birds annually (ca. EUR 20000); and formal report writing and production and associated post reporting activities (ca. EUR 20000). Material costs for transports and lab disposables are minor in the Netherlands, but are occasionally higher if providing volunteer groups with materials such as freezers. The actual field work in this approach is conducted without cost by volunteer beach bird surveyors or other people/organisations regularly surveying beaches. Their reward is provided by the coordinator, who spends a considerable part of her effort on providing good reports to the participants about the programme's outcomes (through reports, the web page, individual contacts).

In the Dutch programme no funds are allocated to assisting other countries, integrating data analysis or report writing for the OSPAR Commission (e.g. for its intermediate assessments). These tasks are considered incidental and are funded separately. Costs for separate national programmes may be reduced significantly if integration of analyses and reporting by a single lead partner is more structurally arranged and financially supported.

8.3.6 Quality assurance / quality control

The methodology referred to in this tool is based on an agreed OSPAR methodology which has been developed over a number of years with the ICES and the OSPAR Commission and which has received full quality assurance through publication in peer-reviewed scientific literature (van Franeker et al., 2011; Van Franeker and Law 2015). The EcoQO methodology has been fully tested and implemented on northern fulmars (*Fulmarus glacialis*), including those from several North Atlantic and Pacific populations (e.g. Mallory,

2008; Provencher et al., 2009; Nevins et al., 2011; Avery-Gomm et al., 2012, 2018; Kühn and van Franeker, 2012; Bond et al., 2014; Donnelly-Greenan et al., 2014; Trevail et al., 2015; Herzke et al., 2016; Poon et al., 2017; Terepocki et al., 2017), allowing wide spatial comparisons of marine litter in European waters and other North Atlantic and Pacific regions. All methodological details can be applied to other tubenose seabirds (Procellariiformes) with no or very minor modifications. Trial studies have been conducted using shearwaters from the more southern parts of the North Atlantic and Mediterranean, but currently it has proved too complicated to obtain a good regional spread in annual samples. In other seabird families, methods may have to be adapted, as stomach morphology, foraging ecology, and regurgitation of indigestible stomach contents differ and can affect methodological approaches.

8.3.6.1 Trend assessment

In the fulmar assessment, the statistical significance of trends in ingested litter, that is, plastics, is based on linear regression of ln-transformed data for the mass of litter (of a chosen category) in individual stomachs against their year of collection. Recent trends are defined as being derived from all data over the most recent 10-year period. The fulmar assessment focuses on trend analyses for industrial plastics, user plastics and their combined total. Generalised linear model (GLM) procedures using annual frequencies of occurrence were recently applied for modelling expected compliance with the OSPAR target in the future.

8.4 Protocol for litter ingestion by sea turtles

8.4.1 Protocol name

MSFD protocol for the monitoring of litter ingested by sea turtles (*Caretta caretta*) and MSFD protocol for sampling litter excreted by live sea turtles (faecal pellet analysis) (optional).

8.4.2 Protocol description

The gastrointestinal contents of dead loggerhead sea turtles *Caretta caretta* (Linnaeus, 1758) are used to measure trends and regional differences in marine litter ingestion.

The original methodologies were first proposed in Italy and incorporated into the MSFD guidelines (Matiddi et al., 2011; Galgani et al., 2013), and then later applied along the Spanish (Domenech et al., 2018), French (Darmon and Miaud, 2016) and Italian (Camedda et al., 2014) coasts and validated by Matiddi et al. (2017). Finally, the protocol was consolidated in the framework of the European project INDICIT (project number G.A. 11.0661/2016/748064/SUB/ENV.C2) and harmonised with the Specially Protected Areas / Regional Activity Centre (SPA/RAC) protocol (INDICIT consortium, 2018). The procedures for dead sea turtle dissection, including the analysis of ingested litter and possible scenarios for thresholds, have been specified in detail and published as a video tutorial by Matiddi et al. (2019). The protocol proposes the collection of a series of basic and optional parameters. The basic parameters correspond to the minimum parameters fundamental to monitor criterion D10C3 based on the occurrence of litter ingestion and the quantity of ingested litter in sea turtles. The optional parameters allow for the acquirement of more knowledge on the impacts of litter ingestion on an individual's health.

8.4.3 Related marine compartments

Caretta caretta feeds in the water column and on the seafloor. Therefore, these two marine compartments are addressed when quantifying litter in the gastrointestinal tract of loggerhead turtles. The ingested plastics mostly consist of macroplastics, while mesoplastics and microplastics could generally be considered as created through the breaking up of macroplastics during feeding activities.

8.4.4 Technical requirements

As the loggerhead sea turtle (*Caretta caretta*) is a protected species, only authorised people can handle live and dead animals or parts of them. Upon finding an animal, its management and recovery should be reported and coordinated with the responsible authorities. Note that a Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) permit is required if a specimen or sample has to be sent/received.

To minimise risks of infectious diseases such as zoonosis, sanitary precautions for the handling of dead or live wild animals must be followed.

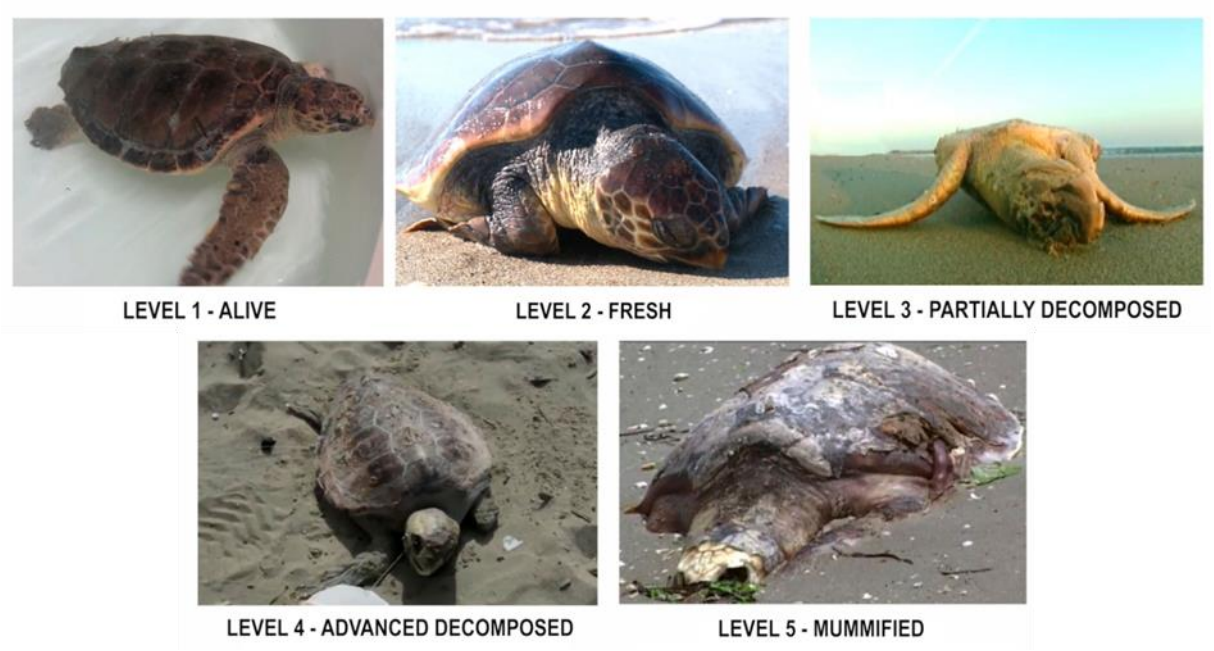
8.4.4.1 Protocol for application in the case of finding a dead sea turtle

Based on initial observations and ideally while still at the place of discovery, some data should be recorded (an observation sheet is provided in Annex VIII – 'Observation sheet for litter ingestion by sea turtles').

A photo of the animal should be taken before any manipulation.

The specimen's body condition level should be reported on the following scale: 1 (alive), 2 (fresh – dead recently), 3 (partially decomposed – internal organs are still in good condition), 4 (advanced decomposition – skin scales are raised or lost) or 5 (mummified – part of the skeleton or part of the body are missing) (Figure 8.1). For level 1, litter can be extracted from the analysis of faeces in a rescue centre. Levels 2 and 3 are adequate for litter ingestion analysis from necropsies. Level 4 allows the measurement of biometric data and assessment of the presence/ absence of ingested plastic (for the evaluation of the frequency of occurrence of litter ingestion (or prevalence, expressed as a percentage – FO %) and entanglement. Level 5, for which individuals have usually lost the gastro-intestinal material, the analysis of litter ingestion is not possible.

Figure 8.1. Specimen's body condition level



Source: Modified from Matiddi et al. (2019).

The circumstances of the animal should be noted based on four categories: stranded (animal found on the beach or on the shoreline); bycatch/fisheries (animal captured actively by fishers, for example ingestion of a hook, trapped in a net, brought back by fishers); found at sea (animal discovered on the sea surface); dead at the recovery centre (the animal arrived alive, but died during its recovery).

The animal should be transported to an authorised service centre for necropsy. In cases where the body is too decomposed for this, the integrity of the digestive tract should be assessed before disposal at the licensed contractor. If the necropsy cannot be carried out immediately after recovery, the carcass should be frozen at –16 °C, in the rehabilitation facility.

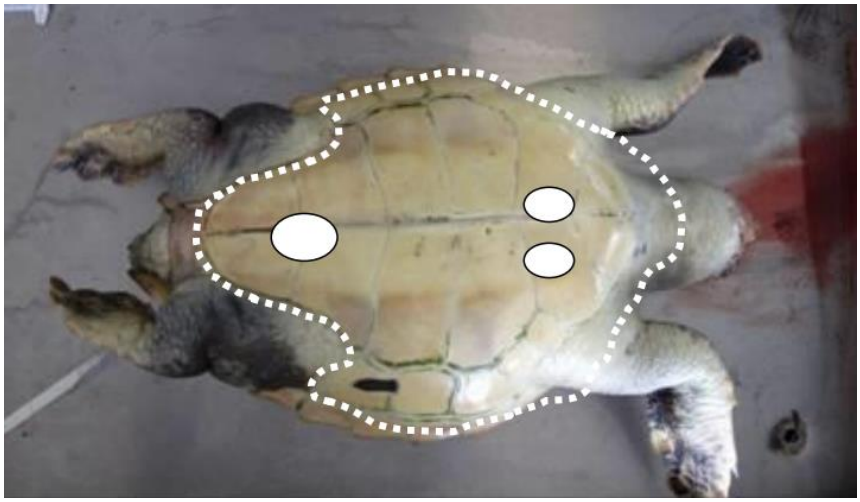
Before the necropsy operation, morphometric measurements should be collected.

The standard curved carapace length (CCL) (notch to tip) (Bolten, 1999) is mandatory, while other measurements are optional (e.g. curved carapace width, weight).

External examination of the animal should be conducted, including inspecting the oral cavity for the possible presence of foreign material. To remove and separate the plastron from the carapace, an incision should be made on the outside edge, as shown by the dashed line in Figure 8.2.

The ligament attachment of the pectoral and pelvic girdle should be cut once the inside of the animal is accessed, as indicated in the white circles in Figure 8.2. Qualitative evaluation of the trophic status of the animal should be made, including the atrophy of the pectoral muscles (none, moderate, severe), and the fat thickness in the articular cavities and on the coelom membrane (abundant, normal, low, none).

Figure 8.2. Cutting line (dashed line) and location of main plastron ligaments (ovals) in a turtle

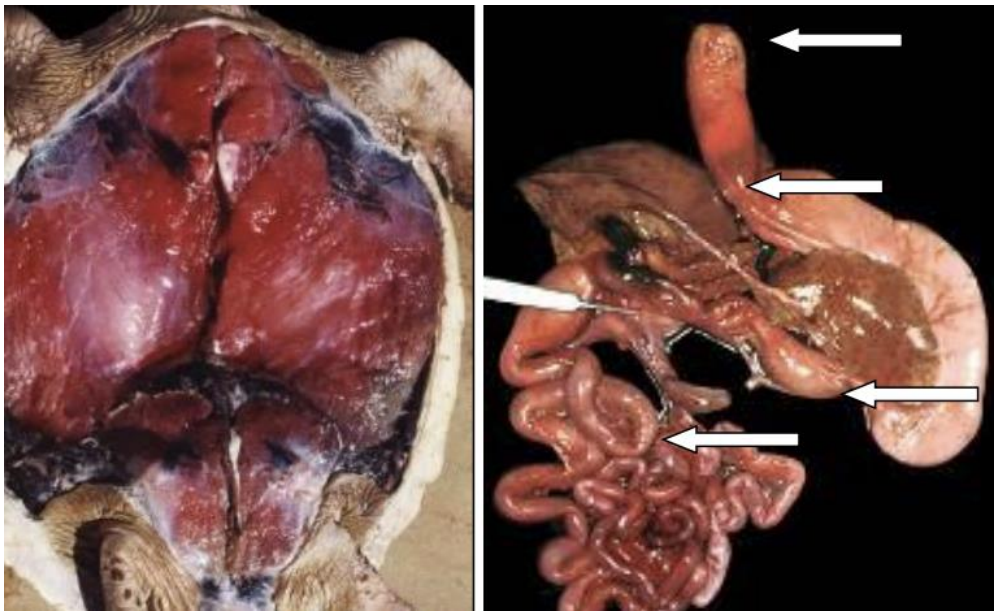


Source: Modified from Wyneken (2001).

Removal of the pectoral muscles and the heart should expose the gastrointestinal system (GI) (Figure 8.3(a)). The different portions of the GI should be isolated by means of plastic clamps, fixed on the oesophagus proximal to the mouth, on the oesophageal valve, on the peg and on the cloaca, as close as possible to the orifice, as indicated by the arrows in Figure 8.3(b). The entire GI should be removed and placed on the examination surface. This is easier if done by at least two operators: one person keeps the animal lying on its side, while the other separates the ligaments of the different organs and the membranes of the carapace by extracting the GI from the animal. The sex of the animal should be recorded. The three parts of the GI (oesophagus, stomach, intestines) should be separated, affixing additional clamps at the cut edges to prevent spillage of the contents.

Figure 8.3. (a) The ventral pectoral and pelvic musculature, which covers most of the internal organs and must be removed to expose the peritoneal cavity and (b) a different portion of the sea turtle GI

(a) (b)



NB: In part (b), arrows indicate location of clamps.

Source: Modified from Wyneken (2001).

The following sampling procedure of GI contents can be applied to any section of the GI (oesophagus, stomach, intestines).

The section of the GI should be observed and any ulcers or any lesions caused by hard plastic items should be recorded.

The contents should be inspected for the presence of any tar, oil, or particularly fragile material that must be removed and treated separately. The liquid portion, mucus and the digested unidentifiable matter should be removed, by washing the contents with freshwater through a 1mm filter mesh, followed by a rinse of all the material collected by the filter using 70 % alcohol and finally by another rinse in freshwater. The retained content should be enclosed in plastic bags or pots, labelled and frozen, not forgetting to note the sample code and corresponding section of the GI. The contents can then be sent for analysis.

Note that if the contents are stored in liquid fixative, a note must be taken of the compound and the percentage of dilution, which should be communicated to the staff in charge of further analysis.

For the analysis of GI contents, the organic component should be separated from any other items or material (marine litter). The fraction of marine litter should be analysed and categorised according to the shape of the items by using a stereomicroscope (Figure 8.4, Table 8.2). Detailed information on categorisation of marine litter of this type is provided by the INDICIT consortium (2018) and Matiddi et al. (2019).

Table 8.2. Classification of marine litter items plus food remains and natural non-food remains.

Type	Code	Description
Industrial plastic	IND PLA	Industrial plastic granules, usually cylindrical but also sometimes oval, spherical or cubical shapes
Use sheet	USE SHE	Remains of sheet, from bags, cling film, agricultural sheets, rubbish bags, etc.
Use thread	USE THR	Threadlike materials, pieces of nylon wire, net fragments, woven clothing, etc.
Use foam	USE FOA	All foamed plastics, polystyrene foam, foamed soft rubber (as in mattress filling), etc.
Use fragment	USE FRAG	Fragments, broken pieces of thicker types of plastics; can be a bit flexible, but not like sheetlike materials
Other use plastics	USE POTH	Any other type of plastics, including elastics, dense rubber, cigarette filters, balloon pieces and soft airgun bullets
Litter other than plastic	OTHER	All non-plastic rubbish and pollutants
Natural food	FOO	Natural food for sea turtles (e.g. pieces of crabs, jellyfish, algae)
Natural no food	NFO	Anything natural that cannot be considered normal nutritious food for sea turtles (stone, wood, pumice, etc.)

Source: *Adapted from* INDICIT (2018).

Figure 8.4. Examples of marine litter categories: (a) IND PLA, plastic pellets and granules, (b) USE SHE, materials such as plastic bags, agricultural sheets or plastic foil, (c) USE THR, ropes, filaments and other threadlike materials, (d) USE FOA, such as polystyrene foam or foamed soft rubber, (e) USE FRA, fragments of hard plastic material, (f) USE POTH, any other plastic items, including elastics, dense rubber, balloon pieces and soft airgun bullets, (g) OTHER, all non-plastic marine litter, such as cigarette butts, newspapers, rubbish and hard pollutants and (h) FOO, remains of the turtle's natural diet

Source: Matiddi et al. (2019).

The fraction of marine litter and the organic fraction should be dried at room temperature or in an oven at 35 °C for 12 hours. Both fractions should be weighed, including individually weighing the different categories of items identified within the marine litter fraction.

8.4.4.1.1 Extraction of data

Abundance by mass (weight in grams, accurate to second decimal place) is the main information that is useful for monitoring programmes.

Other information that is useful for research and impact analysis includes, the colours of litter items; the volume of litter; the different types of litter; the incidences of different litter in the oesophagus; intestine and stomach; and the incidence and abundance by number per litter category. Other uses of the data set are reported by INDICIT consortium (2018) and Matiddi et al. (2017, 2019).

8.4.4.1.2 Size range

Litter should be ≥ 1 mm (stomach contents are rinsed over a 1 mm mesh sieve).

It is optional to separate microlitter items (1–5 mm) from mesolitter and macro litter items; it is possible to superpose a sieve of 5 mm mesh on the 1 mm sieve.

8.4.4.1.3 Spatial coverage

Dead sea turtles are collected from beaches or at sea; they are often collected because of accidental mortalities, that is, they are victims of longline fishing (bycatch) or of boat collisions, for example.

8.4.4.1.4 Survey frequency

Continuous sampling is required. A sample size of 50 turtles or more is recommended for generating annual averages for the chosen assessment area. For reliable conclusions on change or stability in ingested litter quantities, data over periods of 3–6 years are needed.

8.4.4.1.5 Maturity of the tool

The tool is mature at this stage. Specific monitoring programmes are required. The INDICIT consortium collected more than 1 000 data records, from the international established network, and various countries (Spain, France, Italy) are carrying out national monitoring programmes.

8.4.4.1.6 Regional applicability of the tool

The tool is applicable to the MSFD marine regions where loggerhead sea turtles (*Caretta caretta*) occur, in particular the Mediterranean Sea countries and a part of the Atlantic east coast, but not the Black Sea.

8.4.4.2 Optional protocol for application for sampling litter excreted by live sea-turtles (faecal pellet analysis) in the case of finding a specimen alive

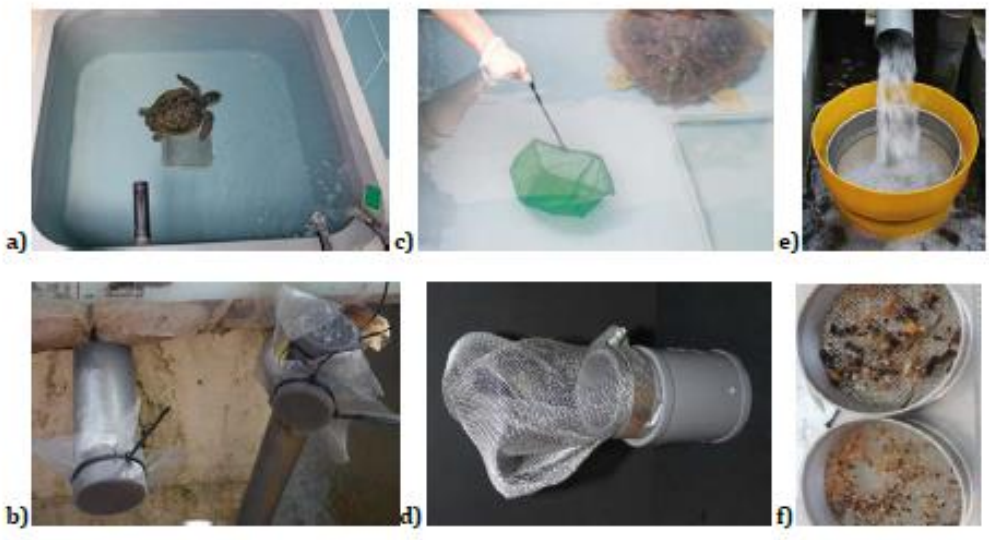
To ensure homogeneity of approaches and allow the comparability of turtles and regions over time, the collected faeces will be analysed only for the individuals remaining in the rescue centre for a minimum of 1 month (Figure 8.5(a)). The faeces are collected for 2 months after the arrival of the individual.

At the rehabilitation facility, the morphologic parameters should be recorded, and the animal placed in the rehabilitation tanks. The standard CCL, notch to tip (Bolten, 1999) is mandatory, while other measurements are optional (e.g. curved carapace width, weight). In most cases, the observed standard time for GI transit is approximately 1.5 months after the first evacuation. The faeces should be sampled from the tank for the entire period of hospitalisation. A 1 mm filter should be placed in all the discharge tubes of the tank (Figure 8.5(b)).

The water tank should be controlled daily by filtering water through the 1 mm mesh sieve according to the following method:

- collect the faeces manually with a 1 mm mesh dip net (Figure 8.5(c));
- put a flexible 1 mm mesh collector in the drain tube (Figure 8.5(d));
- place a rigid 1 mm mesh sieve under the drain (Figure 8.5(e)).

Figure 8.5. Sequence of faeces sampling: (a) the turtle is placed in an individual tank, (b) 1 mm mesh sieves are placed in discharge tubes, (c) a 1 mm dip net for handling faeces, (d) collector with 1 mm mesh placed in discharge tube to filter the water tank (e) a 1 mm mesh rigid sieve down discharge tube to filter the water tank and (f) a sample collected in a rigid sieve



Source: INDICIT consortium (2018).

The digested part of the faeces should be removed by washing the sample with freshwater through 1 mm filter mesh and drying the retained fraction at room temperature. To analyse the litter content and identify the different categories, the same approach as that used for the dead turtle stomach content should be followed and using a similar template.

8.4.5 Estimation of costs

Monitoring activities should be conducted by institutes or rescue centres already authorised and equipped for turtle recovery and necropsy.

A cost estimate for sea turtle litter monitoring is difficult to estimate due to the different national network organisations and the local salary of the involved people. Considering only the average time spent collecting the sample, performing the necropsy, and identifying and analysing the ingested marine litter, monitoring will require:

- at least 50 samples for a country in each subregion;
- two people for 2 days for each sample (200 person-days);
- 3–6 years of monitoring.

8.4.6 Quality assurance / quality control

The previous gaps in QA/QC due to the lack of long-term monitoring programmes have been filled by scientific results in recent years (Camedda et al., 2014; INDICIT consortium, 2018; Matiddi et al., 2011; 2017; 2019).

Specific long-term monitoring programmes are required.

8.5 Protocol for litter ingestion by marine mammals

8.5.1 Protocol name

MSFD protocol for the monitoring of litter ingested by marine mammals (cetaceans and pinnipeds).

8.5.2 Protocol description

The methodology of this protocol follows the methods described in the literature based on the work of responsible bodies for the monitoring of microlitter, mesolitter and macro litter ingested by marine mammals, such as the IWC and ACCOBAMS/ASCOBANS. The amount of macro litter and microlitter in marine mammals can be used to measure trends and regional differences in marine macro litter and microlitter in EU waters and to monitor the impacts of anthropogenic litter on marine mammals and their habitat.

8.5.3 Related marine compartments

Marine mammals hunt and feed in all compartments of the sea: at the surface, in the water column and close to the seafloor (deep-diver cetacean species). Furthermore, they prey on fish of different size classes based on different feeding habits (ranging from filter-feeding species to top predators). As marine litter is affecting marine mammals, no matter in which aforementioned compartments they occur, they all need to be addressed when it comes to macro litter and quantifying litter in the GI tract of marine mammals.

8.5.4 Technical requirements

For marine mammals, impacts from marine litter can be divided into (i) those arising from entanglement in macro litter (see Section 8.8), which can result in injury, drowning or strangulation, and (ii) those arising from ingestion of microlitter and macro litter (both direct and secondary from prey), which can have no effects or having severe direct effects, such as blockage of the digestive tract, suffocation, starvation due to a perceived feeling of satiation and inflammation or even perforation due to sharp objects (Unger et al., 2017; Fossi et al., 2018a). This section focuses on the effects from ingestion. Sublethal impacts include injury, compromised feeding and digestion, associated impacts on malnutrition, disease, reduced reproduction, growth and longevity and generally reduced fitness (McCauley and Bjorndal, 1999; Katsanevakis, 2008; Moore et al., 2013; Werner et al., 2016). While individual strandings provide indications of the range of pathology that can occur, the evaluation of the frequency and severity of impacts of marine litter on cetaceans is complicated. It can be assumed that the number of unrecorded cases is high since devitalised individuals in particular die offshore without reaching the coastline and being available for necropsies. Depending on the presence of a well-established stranding network, the sample size, and thus the detection rate, is low (with only 0–6.2 % of cetacean carcasses recovered from the sea out of the total of estimated mortalities).

A list of suggested species for the monitoring of ingested litter will not be provided here. However, the most representative ones from an ecosystem perspective and from their state of conservation (International Union for the Conservation of Nature (IUCN) – Red List of Threatened Species) should be considered. These may include deep diver cetacean species (*Physeter macrocephalus*, *Ziphius cavirostris*), coastal and pelagic odontocetes (*Tursiops truncatus, Phocoena phocoena*, *Stenella coeruleoalba*, *Delphinus delphis*), mysticetes (e.g. *Balaenoptera physalus, Megaptera novaeangliae*) and several pinniped species.

Ingestion of plastic litter have been documented in over 60 % of all cetacean species, with species employing a variety of feeding techniques in different compartments of the water body (Baulch and Perry, 2014; Kühn et al., 2015; Fossi et al., 2018b). Items ingested are most commonly plastic and range in size from small fragments (< 5 mm) to large plastic items and netting. Pathology can range from no discernible impact to complete obstruction of the digestive tract. When analysing species reported to have ingested marine litter, 50 out of 86 species (58.1 %) had at least one case of ingestion documented (relative to the number of species rather than in terms of the number of individuals being necropsied). Baulch and Perry (2014) stated that a relatively low number of stranding networks are currently established for collecting data on the rates of marine litter ingestion. More recently, Fossi et al. (2018b) published a compressive assessment of more than 86 papers on the impact of the ingestion of marine litter on a variety of cetacean species (Table 8.3).

Table 8.3. Number of cetacean species with documented records of ingested marine litter

	Family	Species total (n)	Ingestion	
			n	%
Baleen whales (*Mysticeti*)	Balaenidae	4	2	50
	Neobalaenidae	1	1	100
	Eschrichtiidae	1	0	0
	Balaenopteridae	8	5	62.5
Toothed whales (*Odontoceti*)	Physeteridae	1	1	100
	Kogiidae	2	2	100
	Ziphiidae	22	14	63.6
	Pontoporiidae	1	1	100
	Monodontidae	2	1	50
	Phocoenidae	7	4	57.1
	Delphinidae	37	19	51.4
	Total	86	50	58.1

Source: Adapted from Fossi et al. (2018b).

The study of microplastic ingestion by cetaceans is a challenging task due to (i) the handling of large volumes of gut contents in particular for large cetaceans, and (ii) the limitation in the availability of precise sample handling when it comes to the avoidance of secondary pollution (Philipp et al., 2020, 2021).

8.5.4.1 Current existing protocols for and approaches to the analysis of the impacts of marine litter in stranded organisms

Protocols for the analysis of marine litter in stranded marine mammals were developed at a workshop hosted by the IWC in 2013; these protocols were recently reviewed according to the existing protocols for other marine taxa (Lusher et al., 2017a, 2018; Fossi et al., 2018b, 2020). A new multidisciplinary approach has also recently been proposed by Corazzola et al. (2021).

In situ examination of entangling and ingested debris and associated traumatic injuries is essential for revealing the pathologic impacts of fishing gear and debris on cetaceans. Impacts can include laceration, amputation and constriction-related injuries externally, and/or blockage, strangulation, ulceration, impaction, emaciation and rupture internally (Unger et al., 2017). Evidence of chronic effects (e.g. emaciation) or prior trauma from entanglement and debris interaction, where material is no longer present, can also be identified as suspected through clinical or post-mortem examinations by scientists. Furthermore, the potential chemical exposure should also be evaluated, which can be accompanied by gross or histologic changes due to the transfer of additives and priority pollutants sorbed from the plastic into the tissues (Rochman et al., 2013; Fossi et al., 2016). Based on the protocols developed during the IWC workshop, recommended procedures are given in the following section for assessing marine litter impacts in stranded cetaceans.

The methodology proposed in this document has already been integrated into the related protocol that was developed by a joint ACCOBAMS and ASCOBANS workshop on harmonisation of the best practices for necropsy of cetaceans and for the development of diagnostic frameworks (Padua, Italy, 24–25 June 2019).

8.5.4.2 Recommended diagnostic approach

To evaluate possible impacts caused by ingestion, a standardised methodology and a classical differential diagnostic approach need to be applied to ensure the comparability of the information collected.

- Investigation of possible traumas, chemical exposure and other sequelae related to the exposure should be conducted.
- Analysis of their role in contributing to morbidity and mortality in the context of other potential causes, such as infectious or non-infectious diseases, nutritional status and other possible ecologies, should be conducted. If a full differential diagnostic approach is not feasible, the documentation of marine litter presence, either external or internal, is still very important. Most studies focus on macroplastics since they are visible and easily accessible. Nevertheless, efforts should also be made to document microplastic occurrence, especially to monitor trends in secondary pollution from prey species.

All necropsies of stranded marine mammals should include the following components, as appropriate.

- Necropsy and reporting. This should include descriptions, sketches, images, measurements, collection, and preservation of entanglement/debris and affected body part(s). The entire gastrointestinal tract should be opened and examined. Standard cetacean necropsy protocols should be followed (McLellan et al., 2004; Pugliares et al., 2007; Moore and Barco, 2013). In the case of microplastic investigations special care needs to be taken to keep the risk of contamination as low as possible.
- Item characterisation. If possible, the object should be named as rope, net, packaging, a cigarette butt or other anthropogenic material. Furthermore, the size (measurement on side) and shape (image analysis of digital photographs) are of importance. If applicable, it is advisable to identify the polymer type of plastics by either Raman spectroscopy or FTIR. All pieces of evidence should be identified using established techniques (Browne et al., 2010) to narrow down the sources and pathways. This information is important for engagement with the relevant industries and sectors, such as plastics and fishing, to establish solutions for minimising the risk of additional litter input into the marine environment.
- Confirmatory diagnostics. To document the presence and the type of items ingested and entangled in, and possible impacts on the animals, further analyses should be undertaken as practical and indicated. This includes histopathology, imaging, analytical chemistry, blood tests and organ function tests. It would be advisable to provide resources to develop techniques for identifying particles of plastic in the tissues of animals. Criteria for the assignation of the degree of confidence of findings (e.g. quality of data) of ingestion or entanglement contributing to or causing morbidity

and mortality have been published and should be applied (Moore et al., 2013). The chain of custody documentation should be maintained as required if applicable.

- Training and database creation. Training designed for specific countries and regions, and establishing a global database and ensuring its maintenance would both enhance the understanding of these problems and help to establish solutions to avoid marine debris input and subsequent impacts.
- Categorisation of contents. The categorisation of GI contents is based on the general morphology of plastic items found, that is, sheetlike, filament, foamed, fragment or other (see list given in Table 8.1). In most cases, smaller fragments will not be unambiguously related to a defined item. However, if possible, items should be described and assigned to a litter category number using the *Joint List of Litter Categories for Marine Macrolitter Monitoring* developed by the MSFD TG ML (Fleet et al., 2021).

For each litter category/subcategory an assessment is made of the:

- incidence (percentage of investigated stomachs containing litter);
- abundance by number (average number of items per individual);
- abundance by mass (weight in grams, accurate to third decimal place).

8.5.4.3 Litter categories – source related information

Categorisation of ingested litter items is essential for understanding their source, distribution, and impact on marine mammals. For marine mammal analyses, stomach contents are sorted into the same categories given above for seabirds (Section 8.3). Following the protocol for seabirds and sea turtles, abundance by mass (weight in grams, accurate to third decimal place) is the main information of use for a standardised monitoring programme. Other information that is useful for research and impact analysis includes, the colour of items, the volume, the different types of litter; the incidences of litter in the oesophagus, intestine and stomach; and incidence and abundance by number per litter category.

8.5.4.4 Size range

Litter should be ≥ 1 mm (stomach contents are rinsed over a 1 mm mesh sieve).

8.5.4.5 Spatial coverage

Dead marine mammals are collected from beaches or at sea; they are often a result of accidental mortalities such as mass stranding (Unger et al., 2016) of bycatch in fishing gear (e.g. victims of longline fishing) or of boat collisions. If not available, the establishment of a national stranding network should be pushed forward and connected at the international level. Furthermore, to establish stranding networks in different countries, it would be advisable to draw on the expertise of countries that already have a stranding network. This helps to keep the data collected consistent and allow for analysis on a global scale.

8.5.4.6 Survey frequency

Continuous sampling is required. A minimum sample size (for the identified species) per year and season must be established in order to draw reliable conclusions on trends or stability in ingested litter quantities.

8.5.4.7 Maturity of the tool

The tool is not mature at this stage. Specific monitoring programmes are required.

8.5.4.8 Regional applicability of the tool

The tool is applicable to the MSFD marine regions where marine mammals suitable for monitoring occur, the Greater North Sea, the English Channel, the Celtic Seas, and the Mediterranean Sea.

8.5.5 Estimation of costs

Owing to the lack of dedicating monitoring programmes at the national level, the cost of monitoring litter on marine mammals is difficult to estimate at this stage. The costs are also related to the dimensions of the species analysed, the proximity to the laboratory where analysis/dissection is carried out, and the cost of

disposal of the carcasses. Cost to be intended per single marine mammal stranding networks in an assessment area and monitoring programmes can be integrated with National stranding monitoring, where available.

8.5.6 Quality assurance / quality control

There is a lack of QA/QC due to a lack of monitoring programmes. The data available are poor quality and based on only a few years (Baulch and Perry, 2014; Kühn et al., 2015 Lusher et al., 2015, 2018; Fossi et al., 2018b, 2020; Corazzola et al., 2021). Only in some cases is it possible to analyse a large time series retrospectively (Unger et al., 2017).

8.5.6.1 Trend assessment

Specific long-term monitoring programmes are required.

8.5.6.2 Target definitions

Specific targets have to be developed, for example, based on the OSPAR Commission recommendation for seabirds (see Section 8.3).

8.6 Protocol for microlitter ingestion by fish

8.6.1 Protocol name

MSFD protocol for the monitoring of microlitter ingested by marine fish.

8.6.2 Protocol description

The methodology of this protocol follows the INDICIT II EU project guidelines for monitoring microlitter particles in the stomachs of marine fish. Background information and technical requirements are described in detail by Matiddi et al. (2021), where the main literature on this topic is also reported. A pilot study evaluating methods and potential sources of bias was conducted during the INDICIT II project by ISPRA (Italy), FRCT (Portugal), CNR-IAS (Italy), EPHE (France), INSTM (Tunisia), HCMR (Greece), EOMAR-ULPGC (Spain), PAU DEKAMER (Turkey), UNIVPM (Italy) and with the results to be published in peer-reviewed scientific literature.

8.6.3 Related marine compartments

Recent studies have highlighted that the feeding habits of different fish species influence microlitter ingestion rates (Lopes et al., 2020) and the analytical methods needed for particle identification (Bianchi et al., 2020). Moreover, the distribution of microlitter items in the marine environment varies according to their shape, size and chemical composition (Palazzo et al., 2021), while several environmental factors (e.g. waves, tides, and currents) on different geographical scales contribute to defining different accumulation pathways for different marine litter types (Angiolillo et al., 2021).

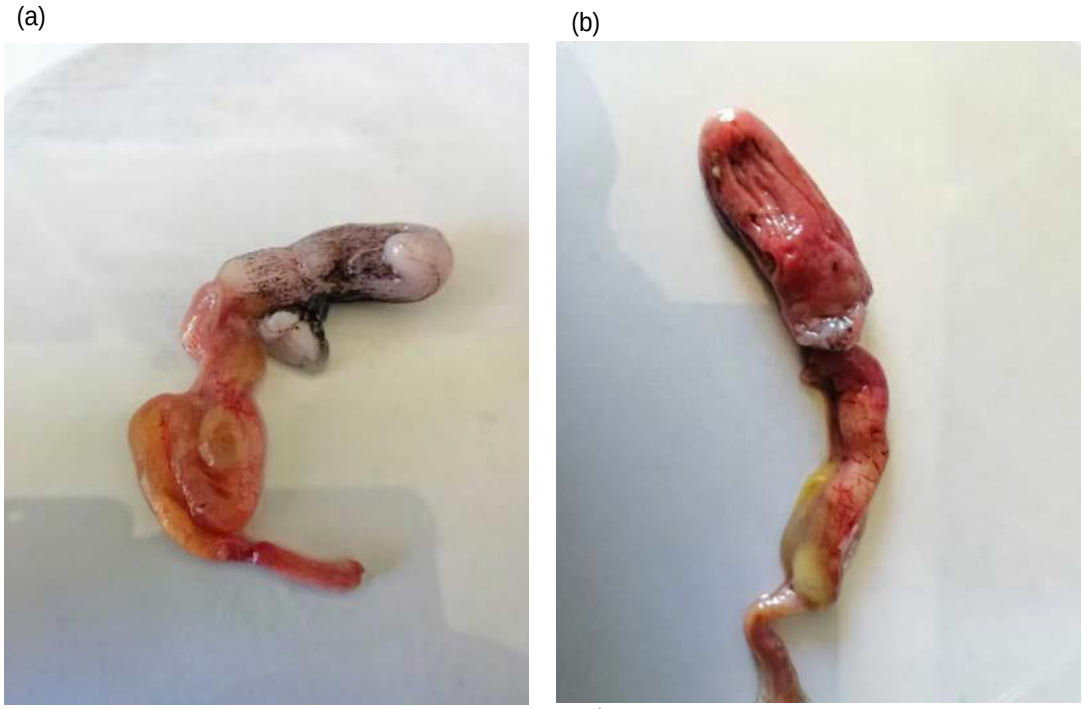
As a result, more than one fish species must be selected for describing the microlitter contamination of the marine environment (Valente et al., 2022; 2023). Some considerations based on previous experiences and recent studies confirm that different fish species are needed to assess all three marine habitat compartments (benthic, demersal, pelagic).

8.6.4 Technical requirements

Samples should be collected and assessed directly on board, checking the fish for any disease and ensuring that any fish showing signs of net feeding or regurgitation are rejected (by checking in the mouth). To avoid any bias due to the regurgitation of plastic items caused by the expansion of the swim bladder, it is recommended to reject all fish with an everted stomach (Figure 8.6) or completely empty stomach (Lusher et al., 2017). All individuals should be rinsed with ultrapure water and frozen upon collection. Samples collected at a fish market or shop are not allowed. Fish can be stored frozen until analysis.

Standardised dissection methods for fish and stomach analysis have been published by the INDICIT II project in dedicated guidelines (Matiddi et al., 2021), and these are summarised here.

Figure 8.6. (a) A normal fish stomach and (b) an everted fish stomach, unsuitable for analysis



Source: Valente, T.

To reduce the possible variability in microlitter ingestion due to differences in the feeding behaviour of fish during different life stages (e.g. juveniles/adults), choosing comparable individuals (e.g. similar size and/or life stage for the species) is suggested.

Several methods and protocols have previously been 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), typically by using potassium hydroxide (KOH) (Box 8.1) or hydrogen peroxide (H_2O_2) (Box 8.2).

Box 8.1. Digestion steps using KOH

Th digestion steps according to Rochman et al. (2015) (modified) are as follows:

— add KOH (10 % weight/volume, 3 × tissue volume) to a beaker;

— optionally, incubate samples using a hot plate, hot bath or oven (≤ 40 °C) to increase digestion speed;

— optionally, neutralise the digestate before filtration by adding 1 M citric acid solution (Thiele et al., 2019);

— use a blank sample to test for possible ambient contamination by adding a similar volume of 10 % KOH as that used in the samples to a beaker without samples (follow the protocol as normal).

The use of enzymes or other methods to degrade bio-organic materials are not reported due to their high costs and the procedural complexity, but they are considered viable alternatives.

It should be noted that both KOH and H_2O_2 could affect plastic particle structures, morphology and colour. For this reason, water baths should be maintained at no more than 40 °C and digestion should not proceed for more than 5 days. It is recommended that the temperature and time of digestion be reduced based on the

organic digestion rate. The use of other reagents is possible, but their potential to cause plastic corrosion should be pre-assessed before analysis (Bianchi et al., 2020; Valente et al., 2022).

Box 8.2. Digestion steps using H_2O_2

The digestion steps according to the MEDSEALITTER project (modified) are as follows:

— for each gram of GI, gradually add 20 ml of H_2O_2 (15 %) into the beaker. Use two aliquots if the GI is ≤ 2 g or more aliquots if the GI is ≥ 2 g;

— optionally, add HNO_3 up to 5 % to increase tissue degradation (Bianchi et al., 2020);

— Incubate samples using a hot plate, hot bath or oven (≤ 40 °C), adding supplementary 15 % H_2O_2 when evaporation occurs, until all organic matter is digested (see Section 8.6.4);

— Add 100 ml of distilled H_2O and stir using a magnetic stirrer;

— Use a blank sample to test for possible ambient contamination by adding a similar volume of 15 % H_2O_2 as that used in the samples to a beaker without samples (follow the protocol as normal).

To standardise the data, pre-filter the solution through a 100 µm sieve, under a laminar flow cabinet, collecting all the material by washing the sieve with ultrapure water. Carefully check the sieve for any possible micro particles remaining. Using a vacuum pump, filter the material retained by the sieve onto a glass fibre membrane, Anodisc or other membrane (i.e. silver, gold) with a mesh size of < 100 µm. Rinse the glass funnel above the membrane with ultrapure water. Place the membrane into a glass Petri dish and cover with a glass top.

Place the Petri dish in a clean cupboard to dry the membrane at room temperature. Detect the number and position of the fibres on the membrane using a stereomicroscope, before opening the dish to avoid airborne contamination during the counting of the fibre microparticles. Note the position of the particles that should be checked. Detect all the other types of microliter items under the stereomicroscope.

The polymer identification is a very important step to distinguish synthetic polymers from any remaining items of natural origin (e.g. organic fibres) and is included in the new Commission Decision (Decision (EU) 2017/848). For example, organic and inorganic particles derived from a natural diet (fish scales or bones, crustacean exoskeletons, etc.) can often be confused with plastics. Spectroscopy techniques offer the most robust polymer identification for suspected microplastic particles, but this requires expensive equipment and is a time-consuming activity that needs personnel with high level of expertise.

Particles of uncertain origin and composition that are longer than 1 mm can be tentatively identified as microplastics using an optical microscope or a hot needle test. However, a minimum of 10 % of the collected items should be analysed and verified using FTIR, Raman spectroscopy (Galgani et al., 2013) or other suitable spectroscopic techniques (e.g. quantum cascade laser spectroscopy).

Textile fibres are ubiquitous, and many laboratories are not well equipped to completely avoid this secondary source of contamination. According to the MSFD TG ML (Galgani et al., 2013), secondary contamination must not exceed 10 % of the results. Avio et al. (2020) proposed that if the blank is contaminated, microlitter items with similar characteristics (shape, colour, 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). Some steps to reduce airborne contamination are reported in Box 8.3.

Box 8.3. How to reduce airborne contamination

The following guidelines are useful to limit levels of contamination:

— close the window and reduce personnel in the laboratory;

— during the procedure of dissection and filtration, process samples under a laminar flow cabinet or glove box (Torre et al., 2016);

— keep the 100 µm sieve clean and protected from air pollution;

— during stereomicroscopy observation of the membrane, cover Petri dishes with a glass dish, cover the stereomicroscope and perform any manipulation under the cover (Torre et al., 2016);

— dress only in cotton clothing;

— use only glass and metal labware, where possible;

— clean all equipment with ultrapure water before each sample analysis;

— perform a blank control at every step, and place a damp filter paper in a Petri dish in the working area to assess any airborne contamination;

— adjust field results according to a blank subtraction approach (Avio et al., 2020).

8.6.4.1 Litter categories – source related information

Even if the new Commission Decision (Decision (EU) 2017/848) only asks for the categorisation of microlitter items comprising artificial polymers, a better categorisation is proposed for data comparison and source identification. A specific template for data collection is proposed in Annex IX – 'Template for data collection for microlitter ingestion by fish' with basic and optional information. Fibres are ubiquitous and generally represent 70-90 % of the total number of microlitter items extracted from fish, but they are not always composed of synthetic material (Avio et al., 2020). Fibres are thought to originate primarily from textiles, and it is currently under discussion if they should be placed in a separate category to filaments (e.g. fishing line) (See also Section 7.4.3). It is also yet to be decided whether beads are to be reported as a single category or included in the category 'granules', with the (smaller) dimension compared with resin pellets. Pellets and granules are also being evaluated as to whether they should be categorised individually.

The following categories, which are based on those first proposed by Kovac Viršek et al. (2016) and later modified by Matiddi et al. (2021), should be used for microlitter ingested by fish.

- Filament. This is a threadlike artificial polymer element that is elongated, generally derived from the fragmentation of fishing gear fragmentation.
- Fibre microparticle, only from textile. This can be short or long, with different thicknesses and colours. It can be made of artificial polymer, be semi-synthetic or be made from natural materials (e.g. wool, cotton, rayon). Note that it is under discussion if fibre microparticles should be categorised in a separate category from filaments.
- Film-layer, foil. This appears in irregular shapes. Compared with a fragment, it is thinner and more flexible. It is derived from sheets or thin films.
- Fragment. This is rigid and thick, with sharp crooked edges and an irregular shape. It can come in a variety of different colours.
- Pellet. This is only from industrial origin. It is usually flat on one side and can be of various colours, be in an irregular or round shape, and is normally bigger in size, around 5 mm in diameter.
- Granule. This comes in a spherical shape, in comparison with a pellet. A granule has a regular round shape and usually a smaller size, around 1 mm in diameter. It appears in natural colours (white, beige, brown). Note that it is under discussion if granules should be categorised in a separate category from pellets.

- Foam. This is flexible microlitter particles in which material cells are all or partly intercommunicating (ISO/TR 20342-7:2021). It most often comes from large particles of plastic foam (including expanded polystyrene and extruded polystyrene foams).

8.6.4.2 Extraction of data

To collect comparable data across different European countries, the INDICIT consortium developed a specific dataset with optional and mandatory information to be collected (Annex IX – 'Template for data collection for microlitter ingestion by fish'). While the main information to be reported is the number of fish with at least one ingested item out of the total number of fish samples, all the other required information is useful for research purposes and for analysis of impact on animal health.

8.6.4.3 Size range

Different definitions of microlitter and microplastics have been proposed:

- 'all sorts of small manmade particles, less than 5 mm in two of the three dimension or diameter, that pass t[h]rough a 5 mm mesh screen but are retained by a lower one';
- '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 sieve but are retained by a lower one', proposing to fix the lower limit for micro items at 100 µm.

To harmonise sample collection and data comparison, microlitter is defined as particles of < 5 mm in the maximum length, excluding fibres **of ≥** 5 mm. The lower limit for monitoring microlitter in biota is fixed to 100 µm.

Alternative size classes have been proposed by Valente et al. (2019) and Matiddi et al. (2021), where the lowest limit is harmonised according to the BASEMAN proposal (Frias et al., 2018) for monitoring microplastic in sediments (100 µm), and the size classes from 330 µm up to 5mm (330 µm ≤ x < 1 mm; 1 mm ≤ x < 5mm) are comparable with data coming from microplastic sea surface monitoring, using manta trawls (Galgani et al., 2013).

To maintain harmonisation within the chapter 7 microliter of this guidance, the size classes proposed are modified as follow (Table 8.4)

Table 8.4. Proposed size classes for marine litter monitoring

Size classes	From	To
Size class 1	1000 µm	4999 µm
Size class 2	300 µm	999 µm
Size class 3	100 µm	299 µm

Source: Modified from Valente et al. (2019) and Matiddi et al. (2021).

8.6.4.4 Spatial coverage

To date, it has not been possible to identify a single target species that is representative for all the MSFD marine waters. Many target species have been proposed for the Mediterranean Sea (Fossi et al., 2018a; UNEP/MAP SPA/RAC, 2018; Bray et al., 2019), deep-water habitats (Alomar and Deudero, 2017; Valente et al., 2019), the Atlantic Ocean (Herrera et al., 2019; Pereira et al., 2020) and the North Sea (Kühn et al., 2020). A wide intercomparison of the Mediterranean Sea, the Black Sea, the eastern Atlantic Ocean and the northern European seas should be planned.

8.6.4.5 Survey frequency

Continuous sampling is required even if differences in ingestion rate in respect of different seasons should be considered. The number of sampling stations must be representative of the entire area assessed (e.g. national

sub-region). The number of collected specimens per sampling station must not be lower than 30 individuals per species, to combine the right levels of effort and statistical analysis (Di Giacomo and Koespell, 1986). Assessment areas and sampling stations should be planned locally according to the heterogeneity of the RSCs.

For very clean areas (i.e. scarce microplastic sources of pollution), it is necessary to increase the number of fish to 50 individuals. Considering that three environmental compartments (i.e. benthic, demersal and pelagic) should be investigated for each area, at least 90 individuals (30 individuals × 3 species) per sampling station must be collected.

8.6.4.6 Maturity of the tool

The tool is not mature at this stage. Specific monitoring programmes are required.

To reduce possible variability in microlitter ingestion due to the variation in the feeding behaviour of fish during different life stages (e.g. juveniles versus adults), it is suggested that comparable individuals be chosen, fixing the fish size around the size of first maturity. However, more studies are needed to investigate the relationship between microlitter ingestion and the ontogenetic stages of different species.

8.6.4.7 Regional applicability of the tool

The tool is applicable to all the MSFD marine regions.

8.6.5 Estimation of costs

To reduce costs associated with sampling, it could be possible to collect samples from ongoing monitoring programmes, such as fish stock assessments cruises (e.g. MEDITS, SOLEMON, ICES-DATRAS, etc.). EU DCF surveys could be used as a platform to conduct sampling of the target species.

8.6.6 Quality assurance / quality control

Specific long-term monitoring programmes are required.

Background contamination is one of the major issues affecting the reliability of ingested microlitter quantification (Prata et al., 2021). It is therefore necessary to reduce airborne contamination with some specific procedures. For example, samples must be processed under a laminar flow cabinet or glove box (Torre et al., 2016). Similarly, during stereomicroscopy observation of the membrane, Petri dishes must be covered by a glass dish. Whenever possible, only glass and metal labware must be used. A blank control must be performed at every step.

Following Avio et al. (2020), field results should be adjusted according to a blank-subtraction approach, where microlitter items with similar characteristics (shape, colour, 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 sample in the same batch).

8.7 Protocol for microlitter ingestion by mussels

8.7.1 Protocol name

MSFD protocol for the monitoring of microlitter ingested by mussels.

8.7.2 Protocol description

The methodology of this protocol follows the methods described in the literature for monitoring microlitter items (< 5 mm) in mussels. The microlitter content in mussel body can be used to measure trends (spatial and temporal) and regional differences in marine microlitter.

8.7.3 Related marine compartments

The tool is proposed for application for benthic filter-feeding mussels, such as blue mussels, oysters and clams in shallow coastal waters (water depth of < 5 m). Therefore, the water column and the seafloor compartments of the marine environment are addressed when quantifying microlitter in the tissue of different mussel species.

8.7.4 Technical requirements

Microlitter in mussels has been investigated in a number of studies (Van Cauwenberghe et al., 2015; Lusher et al., 2017; Karlsson et al., 2017; Catarino et al., 2018; Li et al., 2018; Phuong et al., 2018; Waite et al., 2018; Reguera et al., 2019) and previous European projects focused on harmonising methods to use this organism in microplastic monitoring (Bessa et al., 2019). To date, however, there is no agreed protocol for sampling and subsequent laboratory analyses. Compared with the monitoring of motile marine animals, the monitoring of microlitter in mussels is advantageous, because mussels can be used and sampled with low logistic and financial efforts. Alternatively, where mussels are abundant over long periods, exposure in cages is unnecessary, and mussels can be sampled directly from the sea.

The following species are proposed as potential indicator species that cover the North and Baltic Seas, the north-east Atlantic Ocean, the Mediterranean and the Black Sea: blue mussels (*Mytilus edulis* (L.)), Mediterranean mussels (*Mytilus galloprovincialis* (L.)) and European flat oysters (*Ostrea edulis* (L.)). Further species can be considered for microlitter monitoring (i.e. the Baltic clam (*Limecola balthica* (L.))), which is an infaunal bivalve, living buried in the mud or silt and extending its siphons to the bottom surface. Through the siphons it feeds on organic matter on the sediment surface. Therefore, monitoring microplastics in *Limecola balthica* can provide information about microplastic ingestion from the sediment surface.

It is recommended that mussels be deployed in cages for 3–4 weeks (Catarino et al., 2018) to achieve a steady state of microlitter concentrations in the mussels between feeding and excretion. The cages should be fixed to the sea bottom, and the positions are recorded by means of a GPS. It is recommended that the cages not be marked with buoys, as this can lead to removal of the cages by fishers.

The mussels should be from natural populations from the region where the monitoring is being conducted. The depth should lie between 3 m and 5 m for blue mussels, and each cage should contain five to six specimens, which should subsequently be pooled for analyses in the laboratory (Lusher et al., 2017). A larger number of individuals is necessary for mussel species smaller than blue mussels. To cover the small-scale spatial variability of microlitter concentrations in seawater and mussels, there should be at least three replicate cages at each location.

Ideally, water adjacent to the cages should be sampled and subsequently analysed for microlitter in parallel to the mussels. Parallel sampling allows for bivariate correlation analyses between concentrations in the two compartments. A significant good positive correlation would provide evidence that the mussels are appropriate indicators of microlitter pollution in ambient seawater. It is recommended that the sample water be sampled at least two times during deployment (i.e. at the beginning and at the end of the deployment period). Water sampling is done using a vacuum filter pump and a micro-fibre filter (grade GF/D, 2.7 µm) from onboard a boat (Lusher et al., 2017). The suggested volume of filtered water is approximately 1000 l, which should ensure a sufficiently high abundance of microplastic particles.

After deployment, the mussels are sampled and transported to the laboratory in a cool and moist state. In the laboratory, the size of the individual mussels is determined, the shells and the byssus filaments are removed, and the wet weight of each mussel pool, consisting of the tissue of several individuals, is determined (accurate to the fourth decimal place). Afterwards, the tissue is frozen, pending digestion of the samples. Alternatively, directly after transport to the laboratory, the mussels can be frozen pending further treatment and analysis.

Sample treatment and analyses follow the recommendations of Lusher et al. (2017), who performed investigations on microplastics in blue mussels in Norwegian marine waters. In the laboratory, the water filtrates and pooled mussel tissue (i.e. of five to six individuals for blue mussels) are treated with 10 % KOH solution in glass jars. The glass jars are incubated in an oscillation incubator at 60 °C and 145 rpm for 24 hours. Subsequently, vacuum filtration is carried out using glass fibre filters. Afterwards, the filters are dried at room temperature for 72 hours prior to analyses with a FTIR spectrometer.

Enzymatic digestion is a viable alternative to 10 % KOH (von Friesen et al., 2019), but the protocol could be more expensive and not possible for all users.

It is recommended that FTIR spectroscopy be used for analyses, but in the absence of a FTIR spectrometer, other methods, such as Raman spectroscopy or the Nile red method (Maes et al., 2017), can be applied. For every step of sample transport and treatment, blank samples have to be taken to account for contamination, and all lab equipment should be rinsed threefold with filtered water to minimise contamination.

For each litter category, an assessment is made of the:

- abundance of litter items (average number per individual);
- abundance by mass (average number per weight in grams of mussel pools, accurate to the fourth decimal place).

Owing to potential temporal variations, it is recommended to describe 'current levels' as the average for all data from a location from the most recent 5-year period, in which the average is the sample average, also including subsamples that were found to contain no microlitter.

8.7.4.1 Litter categories

The following categories should be used for microlitter ingested by mussels, which are based on those first proposed by Kovac Viršek et al. (2016) for sea surface monitoring and latter modified by Matiddi et al., 2021:

- Filament. This is a threadlike artificial polymer element that is elongated, generally derived from the fragmentation of fishing gear fragmentation.
- Fibre microparticle, only from textile. This can be short or long, with different thicknesses and colours. It can be made of artificial polymer, be semi-synthetic or be made from natural materials (e.g. wool, cotton, rayon). Note that it is under discussion if fibre microparticles should be categorised in a separate category from filaments.
- Film-layer, foil. This appears in irregular shapes. Compared with a fragment, it is thinner and more flexible. It is derived from sheets or thin films.
- Fragment. This is rigid and thick, with sharp crooked edges and an irregular shape. It can come in a variety of different colours.
- Pellet. This is only from industrial origin. It is usually flat on one side and can be of various colours, be in an irregular or round shape, and is normally bigger in size, around 5 mm in diameter.
- Granule. This comes in a spherical shape, in comparison with a pellet. A granule has a regular round shape and usually a smaller size, around 1 mm in diameter. It appears in natural colours (white, beige, brown). Note that it is under discussion if granules should be categorised in a separate category from pellets.
- Foam. This is flexible microlitter particles in which material cells are all or partly intercommunicating (ISO/TR 20342-7:2021). It most often comes from large particles of plastic foam (including expanded polystyrene and extruded polystyrene foams).

8.7.4.2 Size range

Microlitter is classified into large microlitter (1–5 mm) and small microlitter (< 1 mm). Previous studies revealed maxima in the size distribution of microplastics in mussels of well below 100 µm (Phuong et al., 2018). However, it is costly and difficult to detect microplastic particles of < 50 µm. Therefore, it is recommended that the lower size limit of microlitter in mussels be set to 50 µm.

8.7.4.3 Spatial coverage

For the selection of sampling locations, it is recommended that positions in shallow coastal waters that are remote from any significant sources of microlitter, such as harbours or effluents from waste-water treatment plants, be chosen. This ensures that microlitter concentrations reflect the background, are spatially representative of the water body and, therefore, can be used for comparisons with thresholds and for trend analyses.

8.7.4.4 Survey frequency

Deployment of mussel cages should be done once a year and at the same time of the year, being outside the spawning season for mussels. In temperate regions (i.e. the North Sea, the Baltic Sea and the north-east Atlantic Ocean), monitoring should ideally be performed at the end of summer. In the Mediterranean, sampling should be carried out in spring. Monitoring at the same temporal intervals avoids autocorrelation and bias in trend analyses, in turn evoked by seasonality in the growth and in the feeding rates of mussels.

8.7.4.5 Maturity of the protocol

The protocol is not mature at this stage. Specific monitoring programmes are required. For harmonisation of protocols, methods of trend analyses are recommended to follow statistical analyses applied for the OSPAR EcoQO 'northern fulmar'.

8.7.4.6 Regional applicability of the protocol

The protocol is applicable in coastal waters. The selection of species should be optimised for regional comparison. Wherever possible, overlapping species must be chosen in adjacent areas.

8.7.5 Estimation of costs

The most significant costs arise from sample digestion and clean-up, and from FTIR analyses. The overall estimated costs for one FTIR sample amount up to 1 person-day.

8.7.6 Quality assurance / quality control

The methodology needs to be further developed. At present, there is a considerable lack of QA/QC due to the non-existence of long-term monitoring programmes. The mussel microlitter studies mentioned above have generated only a small amount of data, mostly representing 'snapshots' and which do not currently allow for trend analyses.

8.7.6.1 Trend assessment

Due to the lack of maturity of the tool, specific long-term monitoring programmes have to be developed, generating the sufficiently long time series necessary for trend analyses.

8.8 Entanglement of sea turtles and marine mammals

Different methodologies could be used for monitoring the rate of entanglement. Stranding and photo identification networks or drones are some examples of ways to obtain data on entangled marine animals. The main reason for the lack of data is that in Europe, a great part of marine megafauna (all sea turtles, certain seabirds, and marine mammals) are protected species and their handling require specific permits from national/regional authorities in accordance with applicable regulations. It is challenging to engage stakeholders in data sharing without established conventions and specific agreements. For this reason, the best way to collect data is from official stranding networks or recovery centres, using the same networks involved in the collection of data on marine litter ingestion by sea turtles.

In general, stranding networks make a continuous and almost homogeneous efforts year by year, creating an important source of data about marine fauna threats and impacts. A multitude of professionals and experts are engaged in this process (veterinarians, biologist, environmental authorities, etc.). Multiple parameters are collected to describe the circumstances of each stranding event. Rescue centres are usually associated with or coordinated by stranding networks, so detailed and accurate data about each stranded animal are usually collected.

Another way to collect data is through the activity of citizen science (online platforms), where images are collected by the general public or environmental organisations. This kind of data are not homogeneous, and it is necessary to involve experts to check and catalogue the information from images.

Two protocols are presented to collect data on the entanglement of sea turtles and marine mammals:

- the protocol for the collection of entanglement data from stranding networks or recovery centres (the standard protocol);
- the protocol for the collection of entanglement data from citizen science, with data and images collected by the general public and environmental organisations (the social media protocol).

8.8.1 Entanglement data on sea turtles and marine mammals gathered from stranding network or recovery centres

8.8.1.1 Protocol name

MSFD protocol for the monitoring of entanglement of sea turtles and marine mammals from stranding network or recovery centres.

8.8.1.2 Protocol description

This protocol to assess entanglement of sea turtles and marine mammals gathered from stranding network or recovery centres was drafted after the TG ML meeting on harm, held at Berlin in 2019, and the Joint ACCOBAMS and ASCOBANS Workshop on harmonisation of the best practices for necropsy of cetaceans and for the development of diagnostic frameworks, which was held in Padua, Italy, on 24-25 June 2019. Several steps were carried out to update this entanglement monitoring protocol and related assessments during the implementation of two European projects: the MEDREGION project (Silvestri et al., 2021) and the INDICIT II project (Loza et al., 2021), the protocols for the collection of data were defined and the collected data were aggregated.

The aim of this protocol is to provide an easy tool for comparing harmonised data and comprehending the impact of marine litter on the marine environment, either globally or on a local scale.

The main points of this protocol are the:

- homogeneous effort;
- data quality (collected by experts);
- small spatial scale of data, depending on the number of stakeholders involved.

Using this protocol, it is possible to collect homogeneous data to assess the impact of marine litter on marine organisms. Two kind of data could be obtained on marine megafauna: (i) the general data (number of stranded/registered animals per year, number of entangled animals per year), to allow the (FO %) of entanglement per region/area and per year to be obtained, and (ii) the individual data (details of the stranding event, characterisation of the litter, impact of the litter on individuals) to allow analysis of the percentage of marine litter items causing entanglement in marine fauna, and the main injuries and impacts caused by entanglement.

8.8.1.3 Related marine compartments

This protocol primarily focuses on sea turtles and marine mammals; therefore, the water column and especially the water surface or the seafloor are the marine compartments addressed when quantifying entanglement for:

- sea turtles (mainly water surface):
    - loggerhead sea turtle – *Caretta caretta* (mainly oceanic habitats);
    - leatherback sea turtle – *Dermochelys coriacea* (oceanic habitats);
    - green sea turtle – *Chelonia mydas* (neritic habitats);
- marine mammals (water surface and water column):
    - common bottlenose dolphin – *Tursiops truncatus*;
    - striped dolphin – Stenella coeruleoalba;
    - common dolphin – *Delphinus delphis*;
- other species.

8.8.1.4 Technical requirements

In Europe, many marine megafauna (all sea turtles, certain seabirds, and marine mammals) are protected species, and the operations described below will require a permit according to the national regulations,

including related to animal welfare. Furthermore, health precautions should always be taken regarding zoonosis risks.

Upon finding of a specimen (either live or dead), the authorised staff should proceed to make an external examination of the specimen at the time and place of discovery, or after being hospitalised (live) or stored (dead) in authorised facilities. The data sheets proposed in Annex X – 'Data sheet for recording individual-specific data for entanglement of sea turtles and marine mammals', Annex XI – 'Data sheet for recording of entanglement data on sea turtles and marine mammals' and Annex XII – 'Data sheet for recording general data (frequency of occurrence as a percentage) for sea turtles and marine mammals' are designed mainly with boxes to be ticked to help in recording the requested data. In order to complete the data collection, it is recommended to attach any available post-mortem and/or hospitalisation veterinarian report to the sea turtle entanglement data sheet. Taking pictures is essential for documenting the level of impact of entanglement by litter. The pictures should be carefully codified and stored. For better identification of the categories of impact, participants at the TG ML meeting in Berlin on 21-22 May 2019 recommended the preparation of a photographic atlas, which might be continuously updated thanks to the contribution of European teams in charge of sea turtle monitoring.

8.8.1.4.1 Data collectors (stakeholders)

Stakeholders could be all kinds of organisations/institutions in charge of the stranding networks in a region/country, under environmental authority permits, which make homogeneous efforts over time and include trained staff available for data collection (veterinarians, biologists, public staff with environmental backgrounds, experts, trained volunteers, etc.).

Stakeholders are composed of the following groups:

- local/regional/national stranding networks (coordinated by environmental authorities);
- public/private rescue centres in charge of or associated with stranding networks;
- public/private research institutions in charge of stranding networks (under official permits);
- NGOs managing stranding networks under official permits;
- other organisations involved in or collaborating with stranding networks and rescue centres or involved in marine animal colony monitoring (e.g. of seabirds, seals).

Two kinds of data have been included in the standard protocol:

General data (required from each stakeholder). These are used to obtain the FO% of entanglement per region and per year. They include the following:

- area covered (by the stakeholder);
- number of total stranded/registered animals per year;
- number of entangled animals per year.

Individual data and entanglement data. These are used to obtain accurate and extended information on the impact of entanglement on marine fauna. Specific data from each litter typology should be obtained to identify the main types of litter involved in entanglement per region, spatial and temporal variations, the taxa and species affected, and the impact generated.

Several parameters must be collected from each entangled individual; these are split into four sections.

- Stranding even characterisation. This covers date, location, circumstance, etc.
- Individual characteristics. This covers, size, sex, conservation status (if dead), etc.
- Litter characterisation. This is used to classify and characterise the litter involved.
- Litter impact. This is the parameter 'impact severity' developed by the INDICIT II consortium, based on the effect of injuries/lesions caused by entanglement on animal viability.

All data are described in the data sheets disposed in Annexes X, XI and XII.

Entanglement or bycatch?

Assessing the frequency of entanglement in marine organisms relies on the ability to distinguish between bycatch in fishing gear and entanglement (Kühn et al., 2015; Ryan, 2018). Our ability to distinguish between bycatch in fishing gear and entanglement is low. It is challenging to differentiate between active gear and ghost gear for most entanglement events. Certain marine organisms, when caught in active fishing gear, can tear it off, attempting to free themselves; other will move on after being released by fishermen who voluntarily cut the gear. In both cases, these animals may continue to move over long distances with bits of gear entangled around their bodies (Asmutis-Silvia et al., 2017). For this reason, one of the main obstacles encountered when trying to integrate data is distinguishing entanglement in marine litter from bycatch in active fishing gear. When an animal is found entangled in fishing gear, it is difficult to identify the real origin of the event, that is, if the animal has interacted with the fishing gear whilst it was actively in use, or, if the gear was discarded or lost before the interaction with the animal.

To solve this problem, the INDICIT II Consortium decided to establish adequate definitions of entanglement and other related concepts.

- Marine litter (UNEP, 2021). items that have been deliberately discarded, unintentionally lost or transported by winds and rivers, into the sea and onto beaches.
- Ghost gear. Any fishing gear that has been abandoned, lost or discarded in the sea. There are many reasons why fishing gear can be lost or abandoned, including severe weather, snags beneath the surface, conflict with other gear, interaction with other vessels and intentional discard when no other options are available.
- Entanglement (INDICIT II proposal). The process of being wrapped, trapped or stuck in marine litter.
- Bycatch (European Commission). The inadvertent catch of organisms that were not specifically targeted by a fishing operation (e.g. non-target fish species, marine mammals, seabirds) that are either discarded or landed for commercial sale.
- Doubtful cases. When the item trapping the animal is not present or it is not possible to ensure the distinction between entanglement in marine litter and bycatch in active fishing gear. (These cases should be also registered and included in the databases).
- Accidental catch in active structures. The process of being wrapped, trapped or stuck in anthropogenic structures disposed at sea for any other uses than fishing activities (e.g. anchoring structures, signalling structures).

The INDICIT II consortium decided to establish several criteria to help distinguish entanglement in marine litter from bycatch in active fishing gear.

- Criteria to identify entanglement in marine litter.
    - Litter from land-based sources. This covers packing straps, plastic bags, heavy-duty sacks, etc.
    - Degradation of materials. Degraded material indicates that the item is not suitable for use or has not been used for a long time. Therefore, it should be considered litter.
    - Biofouling attached. The presence of attached biota indicates that the item has not been used for a considerable time period. For this reason, active fishing gear rarely present biota attached, except in aquaculture gear.
    - Medium/small animals (turtles, seabirds, seals, small cetaceans) trapped in large fishing gear. Fishers are unlikely to discard a whole piece of large gear due to the bycatch of medium/small animals, and medium/small animals are not strong enough to pull large fishing gear.
    - Mix of different fishing gear or/and other marine litter. Several materials mixed together indicate that they have been circulating for a long time on the surface and are therefore considered litter.
    - Morphology distortion observed on the animal. This is caused by long-term entanglement.

- Criteria to identify bycatch in active fishing gear.
    - Animals clearly caught by the fishing gear. This covers animals accidentally caught during active commercial or recreational fishing, or directly sent/delivered by fishers due to being bycatch found in their own gear.
    - Ingested hook. These animals are bycatch that are then released after cutting the line.
    - Heavy animals (whales) trapped in large fishing gear. Fishers could discard a whole piece of gear if a large/heavy animal is caught. In addition, large/heavy animals are strong enough to pull large fishing gear.
    - Accessory structures of fishing gear (excluding ropes and buoys attached to pots). Animals could be trapped when the gear is working or when it is not, but either involves a direct interaction with active fishing gear.
- Criteria to identify doubtful cases.
    - Animal with typical injuries (flipper lacerations, throttle, etc.) but no material present. Injuries could be caused by active fishing gear or by entanglement in marine litter. In these cases, local scientific expertise could support the identification, or the case could be included as doubtful if distinction cannot be assured.
    - The item trapping the animal is difficult to identify as fishing gear.
    - Any other doubtful case that could not be solved by the rest of criteria (e.g. animal trapped on clean and non-degraded net).
- Criteria to identify accidental catch in active structures (not related to fishing activity).
- Animals entangled in any other structure that are at sea but not related to fisheries (e.g. anchoring structure nets to keep algae blooms, jellyfish protection nets, shark protection nets).

8.8.1.4.2 Extraction of data

According to criterion D10C4, to assess the impact that marine litter has on large marine animals getting caught in it, it is necessary to quantify the number of individuals of each species adversely affected by litter. Therefore, comprehensive data on the population of a species in a specific area and the number of animals affected by entanglement are needed. However, in practice, it is not possible to have this information with any certainty. Therefore, general data, such as the number of stranded or entangled animals per year, is used as a proxy to estimate the FO% in a region or area per year. Each region needs to be analysed separately, and caution should be taken when considering variations in the frequency of occurrence from main threats like accidental capture, entanglement, boat collisions, and human interaction. In Table 8.5 and 8.6 are reported two examples of how to report the data.

Moreover, with individual data (details of the stranding event, characterisation of litter, impact of litter on individuals), it is possible to analyse the percentage of specific litter typologies that affect marine fauna, and the main injuries and impacts caused by entanglement.

Table 8.5. Example of the assessment of entanglements per year

Area	Total number of individuals (total individuals stranded or registered in the area covered)	Number entangled (total individuals affected by entanglement)	FO%
A1	500	200	40
A2	300	60	20
A3	100	10	10

Table 8.6. Example of the assessment of megafauna affected by marine litter per year

Area	Number entangled (total individuals affected by entanglement)	Fisheries and aquaculture, *N* (%)	Land based, *N* (%)	Both sources, *N* (%)	Unknown, *N* (%)
A1	200	100 (50%)	50 (25%)	20 (10%)	30 (15%)
A2	60	27 (45%)	11 (18%)	3 (5%)	19 (32%)
A3	10	2 (20%)	5 (50%)	1 (10%)	2 (20%)

8.8.1.4.3 Litter Categories – source related information

The main categories of debris reported to cause entanglement are proposed based on the INDICIT II litter typologies updated using *Joint List of Litter Categories for Marine Macrolitter Monitoring* by Fleet et al. (2021).

8.8.1.4.4 Size range

The size of litter causing entanglement can range from 10 cm up to several metres or square metres.

8.8.1.4.5 Spatial coverage

Dead and live sea turtles or marine mammals are collected from beaches or at sea; they are often collected because of stranding events, sea observations or accidental captures during fishing operations. All the European countries (and non-European countries such as Tunisia and Turkey); have official stranding networks that collect data reports on stranded animals throughout the whole year.

8.8.1.4.6 Survey frequency

Continuous sampling is required. A minimum sample population size for the year and the period of sampling should be established to ensure reliable conclusions after the development stage of a possible indicator.

8.8.1.4.7 Maturity of the tool

This tool is not mature at this stage. Specific monitoring programmes are required.

Moreover, important advances have been achieved by the INDICIT II project, where most of the data records collected on entanglement by the INDICIT II consortium were on loggerhead turtle (*N* = 2332; 97.53 %). To date, the most accurate data were collected since 2017. Moreover, important bases have been created, and most stakeholders have updated and harmonised their databases and incorporated most of the important parameters described in the INDICIT II – standard protocol for entanglement. Therefore, evaluation of GES scenarios and indicators' constraints could be established more accurately in the next MSFD implementation cycle. Data on other species, such as green and leatherback turtles or other taxa (cetaceans, seals and seabirds), are very interesting and could be collected during the next few years following the standard protocol developed by the INDICIT II consortium.

8.8.1.4.8 Regional applicability of the tool

The tool is applicable to the MSFD marine regions.

8.8.1.5 Estimation of costs

The costs of the monitoring of sea turtles and marine mammals entangled in litter can be integrated within stranding and rehabilitation monitoring programmes. Most of these programmes already monitor the ingestion of debris for both live and dead individuals. It can also be mutualised with other programmes, such as the oceanographic and fishery observation campaigns.

Costs estimates depend on the country, the network organisation, the local cost of materials, and the skills and salaries of the involved staff on the local level.

In general, it is proposed that one or two experts on marine litter and marine fauna be involved as focal points in each country/region to coordinate data collection from stakeholders, harmonise the classification of litter involved in entanglement (review pictures, identify new litter typologies, etc.) and establish connections with national authorities to facilitate the transfer of data for MSFD assessment. To estimate this, costs should be calculated based on an average of 8 hours for two employees in each county/region.

Specifically for entanglement, the inclusion of pictures of individuals in the stranding protocols is the best way to achieve accurate databases, which could be reviewed by experts on marine litter to harmonise and avoid confusion in litter classification. Some tools may support data collection, in particular when a turtle is observed at sea or found stranded or as bycatch, for example phone apps or online platforms (e.g. RedPROMAR app, developed by the Canary Islands government, or ObsEnMer which offers a collaborative platform managed by Cybelle Planèete in France), allowing citizen or institutions (NGOs, rescue centres, stranding networks) to post pictures with date and GPS location.

8.8.1.6 Quality assurance / quality control

There is a lack of QA/QC due to a lack of previous dedicated monitoring programmes. The data available have been reported to be poor and based on non-standardised collection of data (Votier et al., 2011; Barreiros and Raykov, 2014; Kühn et al., 2015; Lawson et al., 2015; Werner et al., 2016; Duncan et al., 2017; Claro et al., 2018; Anastasopoulou and Fortibuoni, 2019). In general, standard data collected from different regions (and stakeholders) by the INDICIT II project (*N* = 2391 entangled animals) are diverse and disperse, with important differences between species and time periods included. The most accurate data on loggerhead turtle were collected from 2017 to date. Moreover, important bases have been created and most stakeholders have updated and harmonised their databases and incorporated most of the important parameters described in the standard protocol for entanglement. Therefore, evaluation of GES scenarios and indicators' constraints could be established more accurately in the next MSFD implementation cycle.

8.8.1.6.1 Trend assessment

Specific long-term monitoring programmes are required.

8.8.2 Entanglement data on sea turtles and marine mammals gathered from activity of citizen science

8.8.2.1 Protocol name

MSFD protocol for the monitoring of entanglement of sea turtles and marine mammals from activity of citizen science.

8.8.2.2 Protocol description

The aim of this protocol is to collect data from citizen science to increase the official data coming from stranding networks. The use and the integration of these kind of data will be decided by the competent authorities responsible of the national data collection. This protocol can be used by environmental organisations, people who travel with sailboats or fishers. During travelling or fishing activities, these groups can find entangled marine animals and collect data and information on the phenomenon. Regarding stranded animals, citizens must inform local authorities and will be followed the protocol of *"Entanglement of sea turtles and marine mammals from stranding networks or recovery centres".*

Regarding this protocol, the main points are the:

- large spatial scale of data;
- non-homogenous effort;
- lack of usefulness for indicator monitoring;
- requirement for experts to analyse images.

8.8.2.3 Related marine compartments

The water column and especially the water surface or the seafloor are the marine compartments addressed when quantifying the entanglement of sea turtles and marine mammals. This protocol is primarily focused on the following sea turtles and marine mammals:

- sea turtles (mainly surface waters):
- loggerhead sea turtle – *Caretta caretta* (mainly oceanic habitats);
- leatherback sea turtle – *Dermochelys coriacea* (oceanic habitats);
- green sea turtle – *Chelonia mydas* (neritic habitats);
- marine mammals (water surface and water column):
    - common bottlenose dolphin – *Tursiops truncatus;*
    - striped dolphin – Stenella coeruleoalba;
    - common dolphin – Delphinus delphis;
- other species.

8.8.2.4 Technical requirements

In order to collect data through citizen science, it is necessary to organise specific training sessions explaining the problem of the impact of litter on marine fauna, the definition of entanglement, how to distinguish between entanglement and bycatch, and how to use the criteria described in the 'protocol of entanglement from stranding network or recovery centres (see Section 8.8.1):

- criteria for identifying entanglement in marine litter;
- criteria for identifying bycatch in active fishing gear;
- criteria for identifying doubtful cases;
- criteria for identifying accidental catches in active structures (non-related to fishing activity).

Annex XIII – 'Entanglement observation sheet – sea turtles and marine mammals' provides the data collection tool used by environmental organisations or the general public. It is also possible to produce an app containing the same information reported in Annex XIII. An image storage tool is essential to better identify and classify the litter causing entanglement as experts on marine litter could use images to evaluate further details: animal size, litter size, size relationship between the litter and the entangled animal and even the impact of the entanglement (main injuries, animal status, etc.). The pictures of each stranding event are essential to improve the description of the event and collect relevant information that is not registered in the moment.

8.8.2.4.1 Extraction of data

It is not possible to obtain the FO% of entanglement or the percentage of marine litter that affect megafauna from data collected by citizen science, because there are no data on the total number of individuals of a given species present in a given area. For this reason, the entanglement of sea turtles and marine mammals' data from activity of citizen science are considered additional information on the phenomenon.

A specific protocol for conducting images searches regarding entanglement on social media and online platforms has been developed by the INDICIT II project (entanglement protocol – social media review). This protocol could be used by experts within each MSFD implementation cycle to improve indicator criteria and verify litter typologies.

8.8.2.4.2 Litter categories – source related information

The main categories of litter reported to cause entanglement are proposed in Annex XI.

8.8.2.4.3 Size range

The size of the litter causing entanglement can range from 10 cm up to several metres or several square metres.

To facilitate data collection on litter size, the INDICIT II consortium has developed a Litter reference size, which could also be used as a reference for animal size.

8.8.2.4.4 Spatial coverage

The spatial coverage depends on the area covered by the observers at seas (citizens or experts) or the accidental captures during fishing operations.

8.8.2.4.5 Survey frequency

Continuous sampling is required. A minimum sample population size for the year and the period of sampling should be established to ensure reliable conclusions after the development stage of this possible indicator.

8.8.2.4.6 Maturity of the tool

Specific monitoring programmes are required.

The INDICIT II project has found important source of data on entanglement on social media and online platforms. Images of 415 entangled individuals were found and analysed from these sources (data from 2003 to 2021).

The review of these images concluded the following.

- The definitions and criteria developed by the INDICIT II consortium to distinguish entanglement from bycatch are very useful in most of the cases, mainly when images are present. However, larger animals, such as leatherback turtles and large cetaceans, present more difficulties when trying to distinguish entanglement from bycatch.
- The list of litter typologies established by the INDICIT II consortium is appropriate for monitoring the litter entanglement of marine fauna. However, the list could be reduced, based on the taxon and the region, to facilitate data collection by stakeholders.
- Important differences have been found regarding litter entanglement in relation to taxon and sea turtle species; this is probably caused by different behaviours and habitat uses.
- Entanglement was more frequently observed in sea turtles, with entanglement of loggerhead turtle being the most abundant (N = 333). Therefore, loggerhead turtles could be proposed as an indicator to monitor entanglement in oceanic habitats in the Atlantic and Mediterranean Basins.
- Few data on green turtles were found on social media (N = 11), but, depending on the standard data, this species could be proposed for use in monitoring neritic / coastal habitats.
- There is a lack of data on loggerhead turtles in the OSPAR region (only 17 cases were found) in relation to the rest of the regions.
- The parameter of impact severity developed by the INDICIT II consortium (based on the effect injuries caused by entanglement have on animal viability) could be used to measure the impact of entanglement and to identify specific litter typologies that potentially induce greater impacts on the animals.

8.8.2.4.7 Regional applicability of the tool

The tool is applicable to the MSFD marine regions.

8.8.2.5 Estimation of costs

Activities of citizen science do not generate costs. Moreover, one or two experts on marine litter and marine fauna could be involved as focal points in each country/region to coordinate data collection on online platforms, harmonise the classification of litter involved in entanglement (review pictures, identify new litter typologies, etc.), and establish connections with national authorities to facilitate the transfer of data for MSFD assessment. These experts could be the same as those proposed for the standard data.

8.8.2.6 Quality assurance / quality control

There is a lack of QA/QC due to a lack of previous dedicated monitoring programmes.

Moreover, the INDICIT II project has found important sources of data on entanglement on social media and online platforms. Images of 415 entangled individuals were found and analysed (data from 2003 to 2021). A specific protocol (entanglement protocol – social media review) has been developed to harmonise data collection on images collected by citizen science (social media and online platforms). This protocol could be used by experts within each MSFD implementation cycle to improve indicator criteria and verify litter typologies.

8.8.2.6.1 Trend assessment

Specific long-term monitoring programmes are required.

8.9 Entanglement in seabird colonies

8.9.1 Protocol name

MSFD protocol for the monitoring of plastic litter as nesting material in seabird breeding colonies and associated entanglement mortality.

8.9.2 Protocol description

Seabirds are apex predators in marine ecosystems and are particularly vulnerable to entanglement in plastics and other marine litter (Votier et al., 2011). Seabirds such as northern gannets (*Morus bassanus*), shags (*Phalacrocorax aristotelis*) or kittiwakes (*Rissa tridactyla*) tend to incorporate marine litter, much of it originating in fisheries, into their nests, at times resulting in entanglement. Depending on the regional occurrence and distribution of breeding colonies, the nesting materials of different species can be assessed for marine litter. In addition, the associated entanglement mortality can be studied. Ideally both components should be assessed in combination. The share of plastic items in nests of certain species of bird can be used as an indicator of the amount of litter in the natural environment in the vicinity of their breeding sites and to assess entanglement risk of animals. The associated entanglement mortality can serve as an indicator of the direct harm caused by the incorporation of marine litter into the nests of breeding colonies.

A protocol has been developed for the survey of plastic litter as nesting material and associated entanglement in seabirds. These surveys of breeding colonies can serve as a powerful indicator regarding inflicted mortality for seabirds due to marine litter. Negative effects can be documented rather easily and clearly compared with the often more indirect and sublethal effects of plastic ingestion, for example.

Another advantage is that a lot of seabird colonies are already regularly surveyed in many European countries to record the number of breeding pairs and/or breeding success. Thus, a protocol on entanglement in marine litter might potentially be filled out alongside existing investigations without too much extra effort.

8.9.3 Related marine compartments

The litter is collected by seabirds for nest construction in the surroundings of the colonies on beaches and the sea surface.

8.9.4 Technical requirements

First, (part of) a colony should be selected that is easily surveyed from fixed viewpoint(s) and for which the borders of the study section or plot(s) can be easily described. If only a part of a colony is monitored, this should be representative of the whole colony and comprise at least 5–10 % of all nests (at least several tens of nests). Subsampling a representative plot can allow the calculation of pollution and entanglement for an entire colony, but this is also a function of frequency of occurrence. If the frequency of occurrence of marine litter and entanglement is low, a large number of nests needs to be monitored to be able to accurately monitor trends.

GPS and ground marks should be used to fix the viewpoint(s) from which observations will be made and ensure that the spot(s) can be easily found again in later years for continued monitoring.

Photographs should be taken and the exact borders of the study plot documented. In principle, an area fully defined by 'natural' borders should be selected, so it is easily reproducible.

A decision should be made on standard dates on which surveys should be conducted. For plastic as nesting material, one survey is recommended and for entanglement (at least) three surveys per breeding season are

recommended. The dates and numbers of surveys need to be documented to supply information on the observational effort. This may allow for subsequent corrections of entanglement rates. Litter as nesting material and as entanglement should be recorded alongside each other.

For entanglement, the first survey should be made prior to or at the beginning of the breeding season, to distinguish new entanglement victims from old entanglement victims still present from the previous year.

The second survey should be conducted during the peak of the breeding season to record the maximum number of apparently occupied nets (AON) and the respective total number of breeding birds for all species in the monitoring plot(s) and for the entire colony. Here, both entanglement and plastic as nesting material should be recorded. The latter enables the calculation of nest litter rates in relation to all active nests within each plot and for the whole colony.

The third survey should be conducted shortly after the fledging of the chicks at the end of the breeding season to receive an estimate of the minimum total number of birds that died of entanglement during the breeding season. Intermediate counts may refine the picture. The surveys for entanglement and nest litter may be combined with surveys of breeding numbers and success.

For the surveys, binoculars or a telescope of fixed type and magnification should be used (standardising the likelihood of observing details in nest structures). When the location and accessibility of the colonies allow, *in situ* observations can be made provided breeding birds are not disturbed.

A detailed count should be made of the number of nests in the study plot and this should be documented with (digital) photographs whenever possible. This helps to ensure consistent monitoring of plots regarding the number of breeding birds, the occurrence of plastic as nesting material, the categorisation of different litter types and the entanglement rates.

A detailed count should be made of the nests that contain visible marine synthetic litter, documenting pollution by using digital photographs whenever possible. The nest litter rate (frequency of occurrence) is assessed as the number of nests containing visible litter divided by the overall number of nests in the study plot.

During in situ counts, it is possible to record the number of items of litter in each nest (e.g. using five classes: 0, 1-5, 6-10, 11-20 and more than 20 items).

Depending on the situation, attempts should be made to specify details of different types of litter – for example, specifying strings, ropes, net (remains), sheets, packaging, fragments or other types – using the standard MSFD categorisation of litter items based on the MSFD TGML *Joint List of Litter Categories for Marine Macrolitter Monitoring* (Fleet et al., 2021). Attempts should also be made to identify sources of litter, for example, fishing, shipping or recreational. To classify the amount of plastic per nest, a four-step system was designed that could be applied from distant observation points (Table 8.7). For the litter category net and net rests, a slightly different approach is used, as it is impossible to distinguish between net rests in a single nest from a distance.

Table 8.7. Classification of categories of litter in the nests of northern gannets

Class	Nets / net rests	String/rope/packaging
0	No nets or net rests in the nest	No string/rope/packaging in the nest
1	Up to one third of the nest is made up of net rests	1–5 pieces per nest
2	One third to two thirds of the nest is made up of net rests	6–10 pieces per nest
3	More than two thirds of the nest is made up of net rests	> 10 pieces per nest

A detailed count should be made of the birds visibly entangled. All species affected should be recorded separately. In mixed colonies, species that do not use plastic as nesting material themselves regularly become

entangled in the litter used by other species. For example, common guillemots (*Uria aalge*) frequently become entangled in the litter used by gannets. The age (adults, immature or chick) and status (if alive or dead) of the species should be recorded. Entanglement should be documented using (digital) photographs whenever possible. Ideally, these counts should be conducted at standard dates, which need to be defined.

The impact level from litter in nests is assessed as the number of dead or dying animals (specified for species and age classes) divided by the overall number of breeding birds in the study plot (entanglement mortality rate). The number of live birds that are cut loose and released should be specifically recorded as such, but should be included in the totals for individuals mortally entangled, because without human intervention they would have died. In general, the extrapolation of entanglement victims and the entanglement mortality rates have to take direct and indirect losses into account. For example, if a parent gannet or guillemot dies due to entanglement, the brood will usually fail, resulting in the death of the chick. Thus, these indirect victims have to be added to the number of chicks observed to be entangled. Moreover, the number of adults entangled is related to the number of breeding birds in a given colony. For entangled chicks, the number has to be related to the number of chicks. Therefore, the average breeding success can be used as a proxy for the number of chicks present in the colony. To calculate the latter, the average breeding successes of gannets, guillemots and kittiwakes (~ 0.7 chicks per pair), fulmars (~ 0.4 chicks per pair) and shags (~ 1.4 chicks per pair) can be derived based on the long-term seabird monitoring programme data from the United Kingdom (JNCC, 2020).

8.9.4.1 Example monitoring survey

In a colony of 1000 breeding pairs (AON) of gannets, 500 nests are surveyed for entanglement (50 %). The 1000 pairs would produce 700 chicks on average (calculated as 1000 × 0.7 chicks per pair). Ten adult birds and 10 chicks are observed to be entangled. Another 7 chicks (10 × 0.7 chicks per pair) are added due to the death of a parent. The extrapolated number of dead adults would be 20 (2 × 10) and the number of dead chicks 34 (2 × 17) as only 50 % of the colony was surveyed. The entanglement mortality rate for adults would be 20 victims / 2000 breeding adults × 100 = 1.0 %. The entanglement mortality rate for chicks would be 34 victims / 700 chicks × 100 = 4.9 %.

Where colonies are intensively surveyed for population monitoring, entanglement rates can also be compared with the number of breeders, the number of chicks, the breeding success, etc.

However, sometimes, three or more surveys over the breeding season may not be possible. In these cases, a survey at the peak of the breeding season to record the number of breeding birds and active nests is needed for both the nest litter rate and the entanglement mortality rate. For the latter another survey shortly after the fledging of the majority of the chicks in the colony is required. This can supply an estimate of the minimum total number of birds killed by entanglement.

If possible, these surveys should be conducted in a number of different plots to provide a measure of local variability (known to be high, for example, in neighbouring shag colonies in France (Cadiou et al., 2011)).

These surveys can be conducted easily without entering study plots and without disturbance or with minimal disturbance of breeding birds. As a general rule for repeated monitoring, it is not recommendable to collect nest structures after the breeding season to quantify proportions of litter included. In many cases, nests are multi-year structures, and removal may negatively affect the breeding of the nest owners and their neighbours in the next season owing to extra efforts to construct a new nest, disputes with neighbours over remaining nests and materials, or the quality of the nest affecting breeding success. This type of work is only recommended as incidental effort in dedicated research projects.

8.9.4.2 Litter categories – source related information

There are issues to be aware of in interpreting results from this type of monitoring.

Different seabird species have different ranges from colonies when looking for nesting material and may use different types of litter as nesting material depending on their species and location.

The litter in nests of northern gannets (e.g. Montevecchi 1991; Votier et al., 2011; Bond et al., 2012) originates exclusively from the sea, whereas kittiwakes also pick up litter from land to use as nesting material (e.g. Clemens and Hartwig, 1993; Hartwig et al., 2007). Gathering litter from land may also apply to cormorants and shags.

Votier et al., (2011) stated that gannets seem to prefer certain types of plastics, such as synthetic ropes, for building nests, relative to the proportions of them found on adjacent beaches. This apparent selectivity needs to be considered if seabirds are used as indicators for measuring trends in certain types of litter.

8.9.4.3 Size range

Detection of all visible litter particles from microlitter to mega litter is possible, with the focus being on macro litter.

8.9.4.4 Spatial coverage

This protocol is designed for application in breeding colonies of seabirds.

8.9.4.5 Survey frequency

In general, well-built nests are found during incubation and during the rearing period. The nest may frequently be more or less destroyed by the young. To investigate the frequency of occurrence of marine litter, the best period is during incubation at the peak of the breeding season (see Section 8.9.4). To determine the entanglement rate, at least another survey after fledging is required (see Section 8.9.4). As standard procedure, (at least) three surveys for entanglement are recommended (before breeding season, at peak breeding season and after fledging).

8.9.4.6 Maturity of the tool

The tool is not fully mature at this stage. It has been tested and shown to produce sufficient and robust data. Based on the protocols used in previous studies and the requirements of the MSFD, a standard protocol has been developed by various international experts working in the field. The protocol is applicable to a wide range of seabird colonies with justifiable effort and can produce reliable and comparable data.

8.9.4.7 Regional applicability of the protocol

This protocol can be applied in all regions where suitable seabird breeding colonies exist. This covers large parts of the north-east Atlantic Ocean, including the North Sea, Celtic Sea, the Irish Sea and the English Channel, where northern gannets breed. It could also be used in waters such as the Mediterranean Sea, the Baltic Sea or the Black Sea, which are breeding areas for species such as cormorants and shags that build litter into their nests.

8.9.5 Estimation of costs

In cases where this protocol can be applied alongside other monitoring or in existing studies (on breeding pairs/success, or any study involving capture/banding of adults and/or chicks), there may be no additional cruise costs required. If dedicated monitoring is carried out just for this reason, 1–3 days (or more) of cruise to the colony with 1–3 days (or more) of fieldwork will be needed; a driver for the boat is also required. At regularly worked colonies, multiple surveys each year are possible. The estimated costs for the monitoring of nest litter and entanglement based on the long-term experience at the seabird colony on Helgoland are presented in Table 8.8. The equipment costs are low, consisting of binoculars/telescopes, which, in many cases, will be part of existing field equipment.

Table 8.8. Overview of workloads for and financial costs of future monitoring of nest litter and entanglement at the northern gannet colony on Heligoland.

Work step	Annual workload (hours/year)	Annual costs (euro/year)	Initial costs (euro)
Equipment (notebook, GPS, binoculars, telescope with zoom eyepiece, tripod, digital single-lens reflex camera with zoom)			11200.00

Work step	Annual workload (hours/year)	Annual costs (euro/year)	Initial costs (euro)
Mapping of nests in the gannet colony on Heligoland (occurrence, amount and type of litter, survey and documentation of entangled birds)	80	3372.80	
Data processing (litter and entanglement)	100	4216.00	
Data analysis (litter and entanglement)	70	2951.20	
Preparation of a short report	50	2108.00	
Committee and public relation work	25	1054.00	
Project meetings	25	1054.00	
Material expenses		500.00	
Travel expenses		1000.00	
Net sum		16256.00	
10 % overhead		1475.60	
19 % value added tax		3369.00	2128.00
Gross sum		21100.60	13328.00

NB: For the calculation of costs, an hourly net cost of EUR 42.16 was used.

8.9.6 Quality assurance / quality control

Having two observers (or even more than two) count independently can produce error estimates. The methodology has been tested using replicate analyses and shows a certain variation between observers. The protocol applied can supply comparable and reproducible data on entanglement rates and nest litter.

8.9.6.1 Trend assessment

Data analysis and trend assessments can be carried out by time-series analyses (found in most statistic packages).

One problem is the longevity of plastic litter in nests as in many locations these materials may persist for many years if they are not blown or washed away by storms, rain and flooding or taken away by humans.

As a result, nests may contain the plastic litter of several breeding seasons, and trends in the indicator values may show delays and thus have functionality for assessing long-term rather than short-term trends. Finally, as indicated variability scales in the indicator need to be assessed (e.g. Cadiou et al., 2011).

8.10 Entanglement on benthic organisms

8.10.1 Protocol name

MSFD protocol for the monitoring of entanglement and other interactions between litter and benthic organisms.

8.10.2 Protocol description

Seafloor imagery technology allows researchers to quantify the abundance and distribution of litter on the seafloor using a standardised approach and, at the same time, to describe and quantify its interactions with and impact on marine organisms. This methodology is increasingly being used because it consists of a non-destructive sampling technique, with many operating hours and direct observation *in situ*. It is suitable for marine protected areas and sensitive habitats and can provide high-resolution data (depending on the optical device) on marine litter. It can be applied effectively at various depths and to all sea bottom types, including complex rocky habitats, where some litter (especially some ALDFG) may be found in abundance.

This protocol is based on peer reviewed international papers (i.e. Galgani et al, 2013, 2018; Melli et al, 2016; Consoli et al, 2018a, 2018b, 2019; Angiolillo, 2019; Angiolillo et al, 2021). It was developed by considering (i) the Italian MSFD protocol (MATTM/ISPRA, 2020) for the monitoring of coralligenous and mesophotic / deep rocky reefs and (ii) the MEDREGION protocol (Silvestri et al., 2021).

8.10.3 Criteria for choosing the survey areas

The protocol could be applied to different areas of investigation, and should primarily be used in areas where the presence of sensitive benthic habitat, such as coralligenous, mesophotic and cold-water coral (CWC) habitats and deep-sea sponge ground, is known. The habitat should be sufficiently extensive, and the visibility conditions (transparency of the water) in the area should make the investigation possible. In addition, areas should be selected to be representative of different environmental conditions in the sub-region and of impacts of different intensities.

8.10.4 Protocol for investigation

The protocol is based on video-imagery techniques and can be carried out through scuba diving in shallow areas or TUCs, ROVs, AUVs and submersibles for deeper waters.

Each methodology applied should be able to provide controlled sampling, precise data on geographical position and depth, high-definition video, and reference points to use as a metric scale to measure the field of view. In each area of investigation, investigators should:

- Acquire morpho-bathymetric data on the seafloor morphology;
- acquire visual data (high-definition and georeferenced videos/photos) along transects where monitoring activities are conducted;
- processing data to assess the extension and condition of the habitat, the litter abundance and the impact on benthic species.

8.10.4.1 Acquisition of morpho-bathymetric data on the survey area

The acquisition of morpho-bathymetric data should be performed using a multibeam echo sounder (MBES), preferably a hull-mounted one capable of acquiring backscatter data. Bathymetric and morphological data have to provide a high level of detail on the seabed sections of interest (digital terrain model (DTM) at the best possible resolution: cells of 1 m × 1 m, or smaller, in the order of centimetres). The use of the MBES is to be considered a priority for monitoring in the coralligenous / mesophotic / CWCs habitats.

8.10.4.2 Acquisition of visual data

Based on detailed morpho-bathymetric data, 3 investigation sites should be identified in each area, and preferably at a distance of no less than 500 m from each other. ROV exploratory paths should be conducted at each site, within which 3 transects will be identified. These transects should be 200 m long and spaced no less than 50 m. The position of the transects should represent the extension (horizontally and vertically), continuity, and the bathymetric range within which the habitat is included. The surveys should be carried out using a georeferenced remote platform (acquiring high-definition photos or videos). Each video and photographic survey should be recorded in line with the WGS84 datum (expressed in decimal degrees to the fifth decimal place: DD.DDDDD°).

The start of the dive is defined as the moment at which the ROV (or other cameras/ vehicles) dives in the seawater. The end of the dive is defined as the moment at which ROV is at surface/on the deck. The start of the transect is defined as the moment at which the ROV is at the bottom and the end of the transect is when ROV leave the bottom (off the bottom).

The survey area is defined by the video transect width and length. The inspected surveyed area results from multiplying the transect length by the visual field (width) of the video. The visual field can be estimated from the laser pointers scale in the video images. The estimation of litter abundance and litter interaction requires the measurement of the surveyed area.

ROVs (or other cameras/vehicles) should be moved along linear transects, in continuous recording mode, at a constant slow speed (e.g. < 1.5 nm/s) and at a constant height from the bottom (e.g. < 1.5 m), thus allowing for adequate illumination and facilitating the taxonomic and litter identification. Each video transect is

analysed through the imaging technique, using the start and end times of the transect at the bottom as references. A visual census of megabenthic species and litter items has to be carried out along the complete extent of each 200 m long transect, including its width (visual field). The survey area inspected can be calculated by multiplying the transect length by its visual field (i.e. 50 cm visual field × 200 m long transect = 100 m^2 of bottom surface covered per transect). The visual field can be estimated using the laser pointers scale in the video images. The estimation of litter abundance and litter interaction requires the measurement of the area surveyed.

8.10.4.3 Procedures for analysing georeferenced video transects and required parameters

8.10.4.3.1 Location and extent of the habitat

The transect of 200 m has to be positioned on a map at a scale of 1:1500 or 1:2000. The presence of hard or soft bottoms, the presence of structuring species and the extent of the habitat should be reported.

8.10.4.3.2 Condition of the habitat and marine litter

For each video transect, the following parameters must be recorded.

- The extent of hard bottom, calculated as percentage of total bottom extent and showing the type of substratum (rocky reefs, biogenic reefs, etc.), should be calculated.
- Species richness (considering only conspicuous megabenthic sessile organisms), that is, the total number of sessile hard bottom megabenthic taxa should be recorded, identified at the lowest taxonomic level possible.
- The number of colonies or individuals of each structuring species (see Annex XIV – 'List of structuring species') should be counted, and the density of each structuring species should be computed for the transect area of the hard-bottom surface (number of colonies per square metre, or number of individuals per square metre).
- Marine litter should be recorded and counted, in order to obtain information about type, abundance and occurrence. Each item should be classified based on the litter type, following the *Joint List of Litter Categories for Marine Macrolitter Monitoring* (Fleet et al., 2021). The total abundance and occurrence of litter items per transect and the abundance and occurrence per each main category of litter should be recorded. The abundance should be expressed as counts of litter items per area surveyed (number of items per square kilometre), considering the entire transect length and width (in a constant field of view of the camera). When it is not possible to estimate the area surveyed (e.g. when lasers are not available), the unit in which marine litter should be expressed is items per unit length (items per kilometre) (mandatory). In the case of points of accumulation, where it is not possible to count the single items, these will be identified as 'litter hotspots'. These can be expressed as number of litter hotspots per kilometre (mandatory), and also as number of litter hotspots per square kilometre (recommended) or number of litter hotspots per survey.
- For each category of marine litter counted and identified, it must also be indicated whether or not it entangles/covers (entanglement) benthic organisms and, in positive cases, which species and how many organisms are involved. The percentage of colonies entangled in lost fishing gear or other marine litter should be calculated for each structuring species. The interactions recorded relate only to macrofauna identified through visual observations, no further investigation on microfauna is required. If it is not possible to identify the organisms at the species level, taxa should be reported or at least the group (gorgonians, coral, sponge, etc.).
- Any type of additional information should be recorded for each item in respect of interaction and impact.
- All data from each video transect should be entered on the seafloor litter monitoring sheet template (Annex XV – 'Seafloor litter monitoring sheet'), adapted within the framework of the MSFD.

8.10.5 Related marine compartments

The seafloor is the marine compartment addressed when quantifying entanglement and interaction with benthic organisms.

8.10.6 Technical requirements

Given that surveys might be performed using video-imagery techniques through scuba diving or ROV classes with different equipment, or other more sophisticated instruments, it is very important to record any extra camera/vehicle characteristics and instrumentation to ensure harmonisation among and between the teams performing surveys.

To record the interaction of litter with biota, the following conditions are suggested:

- the video survey should allow the recording of the precise position of items;
- the reference points to use as a metric scale for measuring the field of view are recommended;
- noting the camera information is recommended.

ROVs (or other camera/vehicle) should be equipped with the following items:

- an underwater acoustic tracking position system (USBL), to provide detailed geographical and depth positions of the ROV along the transects;
- an automatic depth system (auto depth);
- a compass;
- a high-definition video camera or digital camera (at least 1920 pixels × 1080 pixels);
- a high-definition digital camera (optional);
- laser beams at a known distance, to be used as a metric scale (at least two lasers).

8.10.6.1 Extraction of data

According to criterion D10C4, to assess the impact that marine litter has on benthic organisms through entanglement, it is necessary to quantify the number of individuals of each species adversely affected by litter. To do that, values should be known for the population of species present in a given area and the number of entangled organisms. However, in practice, this information is limited to a very small number of studies (Angiolillo and Fortibuoni, 2020): data on entanglement of benthic species is mainly qualitative, and studies are often limited and heterogeneous in space. The effects of marine litter on marine communities and habitats remain poorly known. There is not enough data on the variation in entanglement rates among species, species vulnerability and the frequency of interactions with different marine litter types. Very few studies put fishing effort, bycatch and the entanglement rate into relation to assess their impacts and obtain information on possible implications in terms of populations (Enrichetti et al., 2019, 2020). Moreover, there is a gap in knowledge on recreational fishing impact, which could significantly affect benthic assemblages.

To address the D10C4 criterion for benthic species a first assessment could be made using data on FO%, considering the number of entangled colonies/individuals of a target species in relation to the total number of colonies/individuals of that species.

Moreover, based on litter data, it is possible to analyse the percentage of marine litter (number and type) that affects marine fauna, the percentage of structuring species affected by entanglement and the main injuries caused by entanglement.

8.10.6.2 Litter categories – source related information

The main categories of litter reported to cause entanglement are proposed according to the *Joint List of Litter Categories for Marine Macrolitter Monitoring* (Fleet et al., 2021) (Annex XI).

The identification and correct categorisation of litter items should be facilitated by the use of photos when images will be analysed in the post-processing step. Unknown litter or items that are not on the list should be noted in the appropriate 'other item box' and descriptive detail and/or the source should be included.

8.10.6.3 Size range

All macroscopic litter items > 2.5 cm (longest dimension) should be identified and counted in each transect.

8.10.6.4 Spatial coverage

The tool is applicable to the MSFD marine regions.

8.10.6.5 Survey frequency

Monitoring is based on an opportunistic approach, taking advantage of any survey occurring at any time when the protocol can be applied. Data will then be collected when possible, planning the reporting to fit with the MSFD implementation cycle, on the basis of this occurring every 6 years.

8.10.6.6 Maturity of the tool

The method is mature and in use.

8.10.6.7 Regional applicability of the tool

The tool is applicable to all MSFD marine regions where shallow sea and deep-sea monitoring activities are established.

8.10.7 Estimation of costs

The costs related to seafloor monitoring surveys can vary widely based on the instrument used (scuba, ROV, submersible). There are no additional costs for the application of the protocol. The identification and quantification of interaction with biota is carried out through the post-processing of the videos acquired. Moreover, the protocol may be opportunistically mutualised with other regular surveys (monitoring in marine protected areas, offshore platforms, etc.) or programmes on biodiversity.

8.10.8 Quality assurance / quality control

The adoption of a common protocol will lead to a significant level of standardisation among the countries that apply it as their sampling strategy. Data on litter on the shallow seafloor are collected through protocols already validated for benthic species (Clean Atlantic, AMAre European projects and Plastic Buster Marine Protected Areas (PBMPA), RAMOGE).

8.10.8.1 Trend assessment

Data analysis and trend assessments can be carried out using time-series analyses. Data series have been collected by former oceanographic campaigns using ROVs in European waters; however, a dedicated study is necessary to make them available for MSFD purposes. Furthermore, no standard dedicated protocol was in use at the time of sampling, in particular for deep waters.